Prognostic biomarkers for head and neck squamous carcinoma

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Prognostic biomarkers for head and neck squamous carcinoma

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Abstract

Head and neck cancer is the 6th most common cancer and is generally treated with surgery and post surgical radiotherapy (RT) alone or in combination with chemotherapy (CT). Over 50% of the patients fail to respond to such treatment while suffering the undesirable side effects of radio-chemotherapy. This highlights the need for new strategies to be able to select patients who could benefit from a specific treatment type to spare cost and suffering to the patients. Furthermore, there is need for targeted and effective therapeutics for this disease. TRAIL and Smac mimetics are novel promising anticancer agents. However, their effect on head and neck cancers so far has not been tested.

The main objective of this research was to investigate biomarkers which could predict response of head and neck squamous cell carcinoma (HNSCC) to novel and conventional therapies. This has been conducted using two different approaches:

The first was to test the sensitivity of HNSCC cell lines to two novel anticancer therapeutic agents, TRAIL and Smac mimetics, and further to identify the biomarkers which could predict response of HNSCC cell lines to these drugs. Furthermore, the underlying mechanisms by which these two agents induce apoptosis in HNSCC cell lines were examined. Nine HNSCC cell lines were tested for their sensitivity to either recombinant TRAIL (h-IZTRAIL) and/or Smac mimetic (Smac59). The pathways involved in their apoptotic response were studied using western blotting, enzyme linked immunosorbent assays and FACS analysis. Overexpression and knockdown experiments were used to examine the importance of certain apoptotic regulators in response to TRAIL and Smac59 treatment. All 9 HNSCC cell lines were found to be successfully killed with either TRAIL or Smac59. TNF-α, caspase8, bcl2 and EGFR expression levels correlated with TRAIL/Smac59 sensitivity. There was no correlation between the expression of inhibitor of apoptosis proteins (IAPs) or TRAIL receptors with Smac59/TRAIL sensitivity in HNSCC cell lines tested.

The second part of the study was a retrospective study to assess response of HNSCC to treatment (surgery and/or radiotherapy) by selecting patients who have been treated with surgery and/or radiotherapy and for whom 5 years follow up data was available. For this part of the project, paraffin embedded formalin fixed biopsies from HNSCC patients were used to construct tissue microarray blocks. The expression of a number of genes involved in the DNA damage repair pathway and tumour hypoxia including PTEN, Ku80, Rad51, XRCC1, ERCC1 and HIF-1α was tested using immunohistochemistry staining IHC. The tested genes were found to be expressed at varying levels in HNSCC patients. Associations between the level of the expression and gender, site of origin, tumour differentiation, TNM stage, lymph node metastases, overall and disease free survival and treatment outcome were investigated using statistical analysis programme SPSS. Using univariate analysis, ERCC1 expression was significantly associated with poor outcome to treatment. HIF-1α high expression was significantly associated with short disease free survival and poor treatment outcome. Using multivariate analysis, PTEN expression was significantly associated with poor treatment outcome.

In summary, it could be concluded that HNSCC cell lines could be killed effectively using TRAIL and Smac59 and their sensitivity could be predicted by level of caspase-8, TNF-α, Bcl-2 and EGFR proteins. TRAIL and Smac59 induce caspase-dependent apoptosis in HNSCC cell lines which consequently results in depletion of XIAP. Smac59 induces TNF-α autocrine secretion in Smac59 sensitive cell lines and adding rhTNF-α sensitised Smac59 resistant cells to Smac59. miRNA expression could be a marker for sensitivity of HNSCC cell lines to TRAIL and Smac59, however more investigation is required to validate this finding.

In conclusion, these findings suggest a potential molecular signature that may be able to predict response of head and neck cancers to TRAIL and Smac59 for the treatment of HNSCC. Expression of genes involved in DNA damage repair and tumour response to hypoxia could be potential biomarkers for patient's prognosis and response to treatment.

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List of abbreviations

APS	Ammonium Persulfate
AVPI	Ala-Val-Pro-Ile
BAF	B-cell Activating Factor
BER	Base Excision Repair
BH1-BH4	Bcl-2 Homology domains 1-4
BID	BH3-Interacting Domain death agonist
BIR	Baculovirus IAP Repeat
BPE	Bovine Pituitary Extract
BRUCE	BIR-containing Ubiquitin Conjugating Enzyme
BSA	Bovine Serum Albumin
CARD	Caspase Recruitment Domain
CC	Coiled-Coil
cFLIP	Cellular FLICE Inhibitory Protein
c-IAP1	Cellular Inhibitory of Apoptosis-1
c-IAP2	Cellular Inhibitory of Apoptosis-2
CT	Chemotherapy
DcR	Decoy Receptor
DD	Death Domain
DED	Death Effector Domain
DFF	DNA Fragmentation Factor
DFS	Disease Free Survival
DISC	Death-Inducing Signaling Complex
DMEM	Dulbecco's Modified Eagle Media
DMEM-F12	Dulbecco's Modified Eagle Media-Ham's F12
DMSO	Dimethyl Sulfoxide
DPX	Distyrene Plasticizer Xylene
DR	Death Receptor
ECL	Enhanced Chemiluminescence

EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme Linked Immunosorbent Assay
ERCC1	Excision Repair Cross Complementation group 1
OSCC	Oesophageal Squamous Cell Carcinoma
FACS	Fluorescence-Activated Cell Sorting
FADD	Fas-Associated Death Domain
FCS	Fetal Calf Serum
GA	Gentamicin Amphotericin
HandE	Haematoxylin and Eosin
HIF-1α	Hypoxia Inducible Factor -1alpha
HNSCC	Head and Neck Squamous Cell Carcinoma
HPV	Human Papilloma Virus
HRR	Homologous Recombinational Repair
IAPs	Inhibitor of Apoptosis Proteins
IHC	Immunohistochemistry
IKK	Inhibitor of κB Kinase
IZ-TRAIL	Isoleucine Zipper–TRAIL
Iκ-B	Inhibitor of κB
LB	Luria-Bertani
LZ-TRAIL	Leucine Zipper-TRAIL
Mcl-1	Myeloid cell leukaemia-1
MMR	Mismatch Repair
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAIP	Neuronal Apoptosis Inhibitor Protein
NER	Nucleotide Excision Repair
NF-κB	Nuclear factor kappa-light-chain enhancer of activated B cells
NHEJ	Non Homologous End Joining
NHK	Normal Human Keratinocytes
NIK	NF-*κ*B-Inducing Kinase
NOD	Nucleotide-binding and Oligomerization Domain
NSCLC	Non Small Cell Lung Carcinoma
OD	Optical Density

OMMP	Outer Mitochondrial Membrane Permeabilization
OPG	Osteoprotegerin
PARP	Poly (ADP-ribose) Polymerase
PBS	Phosphate Buffered Saline
PEFF	Paraffin Embedded Formalin Fixed
PMSF	Phenyl Methyl-Sulfonyl Fluoride
pRb	Retinoblastoma
PTEN	Phosphate and tensin homolog deleted on chromosome 10
RHD	Rel Homology Domain
rhEGF	Recombinant human Epidermal Growth Factor
rhTRAIL	Recombinant human TRAIL
RING	Really Interesting New Gene
RIP	Receptor Interacting Protein
RT	Radiotherapy
SCID	Severe Combined Immunodeficiency
SDS	Sodium Dodecyl Sulphate
siRNA	Small inhibitory RNA
Smac/DIABLO	Second mitochondria-derived activator of caspases, direct IAP-binding protein with low PI
TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline-Tween20
TE	Tris-EDTA
TM	Transmembrane
TMA	Tissue Microarray
TNFR	TNF Receptor
TNF-α	Tumour Necrosis Factors-alpha
TNM	Tumour-Node-Metastases
TRAF1	TNF Receptor-Associated Factor 1
TRAF2	TNF Receptor-Associated Factor 2
Ts-IAP	Testis-specific Inhibitor of Apoptosis
UADT	Upper Aerodigestive Tract
UBC	Ubiquitin Conjugating Domain

UV	Ultraviolet
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organization
XIAP	X-linked Inhibitor of Apoptosis Protein
XRCC1	X-ray Repair Cross Complementing 1

1 Introduction

1.1 Head and neck squamous cell carcinoma (HNSCC)

1.1.1 Definition and incidence

Head and neck cancer is the term used to describe a wide range of malignant tumours originating in the upper aerodigestive tract (UADT), (including oral cavity, nasopharynx, oropharynx, hypopharynx and larynx), the paranasal sinuses and the salivary glands. Cancers at different sites have different histopathological types and head and neck squamous cell carcinoma (HNSCC) is the most common. Cancer of the mouth and oropharynx is the 10^{th} most common cancer worldwide, but it is the seventh most common cause of cancer induced mortality (Mehanna et al., 2011).

In 2002, the World Health Organization (WHO) estimated that there were 600,000 new cases of head and neck cancer and 300,000 deaths each year worldwide, with the most common sites being the oral cavity (389,000 cases a year), the larynx (160,000) and the pharynx (65,000) (Boyle and Levin, 2008). In United Kingdom, about 7,800 patients are diagnosed with head and neck cancer every year. The prevalence has risen by more than 20% in the last 30 years (National Health Service website; www.nhs.uk, 2012). WHO projections estimate worldwide mortality figures from mouth and oropharyngeal cancer in 2008 to be 371,000. This is projected to rise to 595,000 in 2030 (Mehanna et al., 2011).

Recently, a marked increase in the prevalence of tonsil and oropharyngeal carcinomas associated with human papilloma virus (HPV) has been observed. In a population-based study, the incidence of HPV positive oropharyngeal carcinomas has increased by 225% between 1988 and 2004, whereas the incidence of HPV negative oropharyngeal carcinomas has decreased by 50% (Chaturvedi et al., 2011).

1.1.2 Pathogenesis

In general, cancer arises through the accumulation of genetic and epigenetic changes in genes acting in cancer associated pathways, causing acquisition of cancer-related phenotypes, including limitless replicative potential, self-sufficiency in growth signals, insensitivity to anti-growth signals, ability to evade apoptosis and ability to invade adjacent tissues and to metastasise to distant organs (Hanahan and Weinberg, 2000). A key issue in HNSCC pathogenesis is that carcinomas develop within large preneoplastic fields of mucosal epithelium consisting of genetically altered cells that are clonally related to the carcinoma and often extend to the surgical margins when tumours are excised and can cause local recurrences and secondary primary tumours (Leemans et al., 2011).

HPV-induced carcinogenesis is primarily associated with the oropharynx. This subsite of the head and neck region includes the soft palate, base of tongue, and tonsils. More specifically, HPV infection is most commonly associated with the palatine and lingual tonsils (Pai and Westra, 2009). The patient characteristics associated with HPV positive carcinomas differ significantly than HPV negative carcinomas. Patients with HPV positive carcinomas are slightly younger, frequently white males, non smokers or infrequent smokers, and moderate or non alcohol drinkers. Conversely, HPV negative carcinomas develop in slightly older, often heavy smokers, and experience an enhanced risk with alcohol consumption (Gillison et al., 2008).

HPV associated carcinomas in tonsil and oropharynx may be genetically distinct though their pathogenesis is not yet clear. They are presumed to arise in a similar way to cervical carcinomas through E6/7 effect on P53 and retinoblastoma (pRb) (Howard and Chung, 2012). Therefore these carcinomas probably show minimal genetic instability compared with smoking induced carcinomas.

1.1.3 Risk factors

Smoking and tobacco products, alcohol use, and infection with human papilloma virus (HPV) are among the major risk factors for head and neck cancer; globally, smoking and alcohol have historically been aetiologies for approximately 42% of head and neck cancer. However, a shift in HNSCC epidemiology in recent decades has been noted, unrelated to tobacco and alcohol and linked to HPV driven malignancies particularly for oropharynx tumours (Cmelak, 2012). Besides the above mentioned exogenous risk factors, certain inherited disorders and also a more general genetic susceptibility predispose to HNSCC (Hopkins et al., 2008).

1.1.4 Histopathological features

HNSCC is divided histologically into three types according to their differentiation status (resemblance to normal surface epithelium); well, moderately and poorly differentiated. This differentiation is assessed in terms of keratinisation, cellular pleomorphism, mitotic activity and nuclear aberrations (Woolgar and Triantafyllou, 2009).

1.1.5 Prognosis

Several factors affect the prognosis of head and neck cancer; the most important prognostic factors are site and TNM (tumour, node, and metastasis) stage. Recently HPV status has been shown to be of significant prognostic importance; HPV positive cases tend to have better prognosis (Gillison et al., 2008).

Molecular markers of prognosis have been studied but none has yet entered routine clinical reporting. Several candidates have been suggested such as p53 but because of inconsistencies across studies, p53 status has not had a conclusive prognostic value. Other studies showed that patients who were positive for vascular endothelial growth factor (VEGF) had double the risk of death. Many other studies associated tumour hypoxia with adverse prognosis, in particular increased expression of hypoxia inducible factor-1α and carbonic anhydrase 9. High expression of epidermal growth factor receptor (EGFR) was linked with poor

prognosis and also correlated with better response to radiotherapy treatment (Silva et al., 2007). Although many biomarkers correlate with recurrence, metastasis and death, none is sufficiently predictive or independent to be used routinely.

1.1.6 Treatment

1.1.6.1 Conventional treatment

Early stage tumours are usually treated with surgical excision or radiotherapy. Both modalities have shown to have similar cure rates but have different adverse effects. Radiotherapy offers better organ preservation but the morbidity associated with radiation therapy for example, xerostomia, loss of taste, life-long risk to dentition and post irradiation sarcoma can be considerable. For advanced squamous cell carcinoma of the head and neck, single modality treatment (surgery or radiotherapy) is associated with poorer outcomes and randomized studies have shown that combined use of surgery and postoperative radiotherapy or combined chemotherapy and radiotherapy offer the highest chance of cure (Bhalavat et al., 2003). Postoperative radiotherapy is recommended when the risk for loco-regional recurrence in the head and neck cancer exceeds 20%. Chemo-radiotherapy is used when surgical margins are positive for tumour or when lymph nodes show extra-capsular extension (Schoder et al., 2009). All treatments for HNSCC are frequently compromised by late stage presentation and co-morbidity, particularly cardiovascular diseases associated with chemotherapy.

Radiotherapy induces DNA damage leading tumour cells to undergo apoptosis (Lima et al., 2012). Tumour radio-resistance, including intrinsic radio-resistance before treatment and acquired radio-resistance during radiotherapy is a main obstacle to the efficacy of radiotherapy; this radio-resistance is associated with disruptions in DNA damage repair mechanisms, apoptosis and cell cycle checkpoints (Xu et al., 2008).

These treatment modalities are usually accompanied by several complications. Following the surgical treatment, patients present with functional problems such as impaired swallowing and speech and voice problems or, following

neck dissection, weak shoulders (Mehanna et al., 2010). Radiotherapy has acute complications including radiation dermatitis, xerostomia, excessive mucous production and painful mucositis and late radiation toxicity including xerostomia, fibrosis of soft tissues, dysphagia, osteo-radionecrosis and post irradiation sarcoma (Denis et al., 2003). This highlights the need for target specific drugs and biomarkers to target treatment in order to improve cure rate and reduce adverse effect.

1.1.6.2 Targeted therapy

Improved understanding of the molecular biology of cancer development and progression has led to the emergence of new therapeutic agents that target specific molecules involved in cell proliferation and apoptosis. Some of these agents have been approved and are currently in use for treatment of patients with different types of cancer. Many other novel targeted agents are still under laboratory and clinical investigation.

EGFR signalling is important in tumourigenesis and disease progression particularly in cancer with epithelial origin. Cetuximab, a monoclonal antibody against EGFR has been shown to reduce patient mortality and increase loco-regional control of HNSCC tumours when combined with radiotherapy. It became the first molecular targeting strategy approved by U.S. food and drug administration for HNSCC in 2006 (Bonner et al., 2006).

Angiogenesis is essential for tumour growth and metastasis, antiangiogenic therapies for example Bevacizumab, a recombinant human monoclonal antibody against vascular endothelial growth factor (VEGF) has been used in combination with radiotherapy and or chemotherapy for treatment of head and neck cancers in phase III clinical trials with 14.6% response rate (Seiwert and Cohen, 2008).

Tumour necrosis factor-related apoptosis inducing ligand (TRAIL, see section 1.2.8) has been shown to induce selective cytotoxicity in several different types of malignant cell lines. In this study the potential of TRAIL as an anti-neoplastic agent for the treatment of HNSCC was investigated. Recombinant

TRAIL is currently used in phase I/II clinical trials for treatment of patients with advanced solid tumours and has a favourable safety records (Herbst et al., 2010).

Smac mimetics (see section 1.2.9) have been investigated since 2004 as potential anticancer targeted therapeutic candidate. They have been tested on several cancer cell lines either as a single agent or in combination with other therapeutic modalities (Chen and Huerta, 2009).

Resistance to apoptosis is believed to be the main cause of treatment failure and cancer recurrence following chemo- and radio- therapy. This resistance can be partially correlated with inherent or acquired genetic and epigenetic changes of the components of the death pathways in the tumour cells (Goh et al., 1995). It is hoped that further understanding of the molecular pathology of HNSCC will lead to novel therapies and improved tailoring of existing treatment modalities for individualised treatment of HNSCC patients (Leemans et al., 2011).

1.2 Cell death

Cell death generally is classified into two forms based on morphological and biochemical criteria: necrosis and apoptosis (Wyllie et al., 1980).

1.2.1 Necrosis

Necrosis is a pathological cell death resulting from extreme cellular trauma. It affects groups of cells rather than individual cells, and evokes inflammation (Darnell et al., 1994). Cells undergoing necrosis fail to maintain proper plasma membrane function; therefore they can no longer regulate osmotic pressure. Consequently, the cells swell and rupture, spilling their cellular contents into the surrounding tissue, resulting in nonspecific cellular destruction that leads to an inflammatory response necessary to remove debris and begin tissue repair (Griffith et al., 2009).

1.2.2 Apoptosis

Apoptosis or programmed cell death was first described in 1972 by Currie and colleagues (Jin and El-Deiry, 2005) and it is both physiological and pathological process. It is characterized histologically by the formation of small, spherical cytoplasmic fragments, some of which contain pyknotic nuclei (Vaux and Strasser, 1996). The nuclear changes are accompanied by fragmentation of the cellular DNA into a ladder of regular bands, the result of random double stranded breaks in the linker regions between nucleosomes (Cao et al., 2001). While these intracellular changes are taking place, the cell membrane becomes ruffled and blebbed in a process called zeiosis. The fate of the apoptotic cell is to be phagocytised by surrounding cells before it ruptures and release its potentially inflammatory contents. Thus, apoptotic cell death is important in tissue homeostasis, by which cell number within tissues and organs is maintained, as it results in the deletion of cells with little tissue disruption (Griffith et al., 2009).

There are two major apoptotic pathways namely the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (Figure 1.1).

1.2.2.1 Extrinsic pathway

The extrinsic pathway is triggered by the activation of tumour necrosis factor (TNF) death receptor superfamily (Fas, TNF-α, TRAIL). These cell surface receptors are activated when cross linked to their ligands. Death ligand stimulation results in oligomerization of the receptors and recruitment of the adaptor protein Fas-associated death domain (FADD) and caspase-8, forming a death-inducing signaling complex (DISC). Inside the DISC, autoactivation of caspase-8 occurs. The activated caspase-8 consequently leads to activation of effector caspase-3, -6 and -7. These caspases stimulate the cleavage of many cellular proteins causing the appearance of the characteristic apoptotic phenotype (Jin and El-Deiry, 2005).

1.2.2.2 Intrinsic pathway

The intrinsic pathway is triggered by DNA damage induced by irradiation or chemicals, growth factor deprivation or oxidative stress. These signals cause outer mitochondrial membrane permeabilization (OMMP) leading to the release of cytochrome c, Smac/DIABLO and other proapoptotic proteins into the cytoplasm. Released cytochrome c assembles with procaspase-9 to form the apoptosome. Within the apoptosome, caspase-9 is activated and leads to activation of caspase-3, which initiates apoptosis execution. The intrinsic pathway could also be activated in response to death receptor activation. This occurs via activated caspase-8 which leads to the activation of BID (BH3-interacting domain death agonist) transforming it to truncated BID (tBID). tBID consequently leads to OMMP (Newsom-Davis et al., 2009). These pathways are summarised in Figure 1.1.

Cells may be divided into two distinct types in response to different pro-apoptogenic agents. In type I cells, activation of death receptors is sufficient to induce apoptosis whilst in type II cells co-activation of the mitochondrial pathway is necessary to induce effector caspase activation. The distinction between type I and type II cells depends on the expression profile of anti-apoptotic molecules as well as differences in the efficiency of DISC formation (Scaffidi et al., 1998).

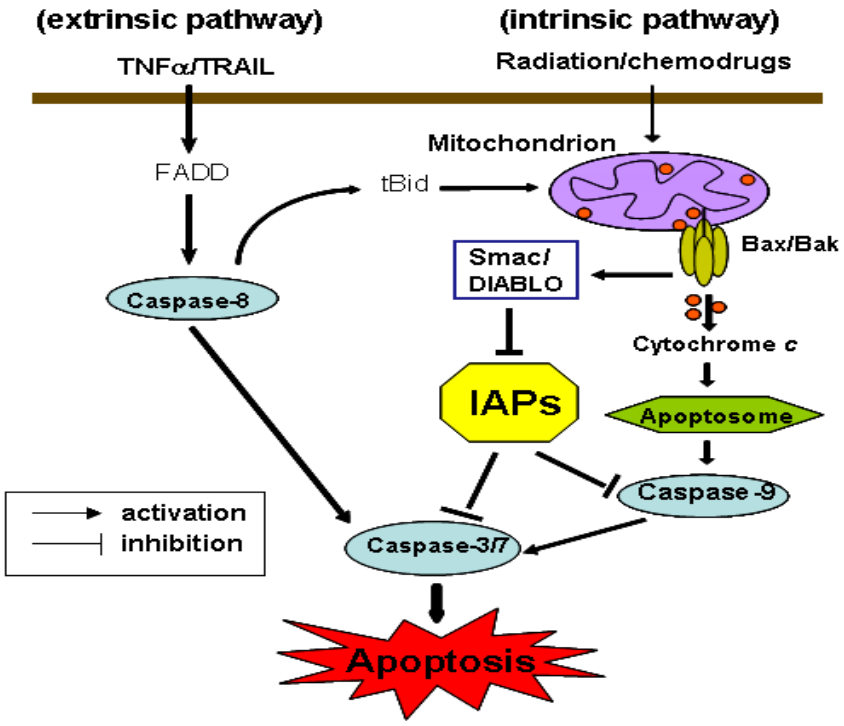


Figure 1.1 Apoptosis pathways in mammalian cells

Apoptosis activation by the extrinsic pathway involves the binding of extracellular death ligands (such as TNF /TRAIL) to death receptors, provoking the recruitment of adaptor proteins, such as the Fas-associated death domain protein (FADD) and recruiting caspase-8. Active caspase-8 then activates effector caspase-3, -6 or -7. In the intrinsic pathway, stresses such as radiation or chemotherapeutic agents target mitochondria and induce efflux pro-apoptotic proteins, such as cytochrome c and Smac/DIABLO , into the cytosol. On release from mitochondria, cytochrome c can interact with the apoptosome assembly. Active caspase-9 then activates effector caspase-3, -6 or -7. Effector caspases stimulate the cleavage of many cellular proteins causing the characteristic apoptotic phenotype. From Dai et al., 2009a.

1.2.3 Inhibitor of apoptosis protein (IAPs) family

Inhibitor of apoptosis proteins (IAPs) is a group of cellular proteins that function as potent endogenous inhibitors of apoptosis. They suppress apoptosis induced by a variety of stimuli, including death receptor activation, growth factor withdrawal, ionizing radiation, viral infection and genotoxic damage. The IAPs family contains eight members; Neuronal apoptosis inhibitor protein (NAIP), X-linked inhibitor of apoptosis protein (XIAP/MIHA/hILP/ BIRC4/ILP-1), cellular IAP1 /human IAP2 (c-IAP1/HIAP2/MIHB/ BIRC2), cellular IAP2/human IAP1 (c-IAP2/HIAP1/MIHC/ API2/BIRC3), Testis-specific IAP (Ts-IAP/hILP2 /BIRC8 /ILP2), BIR-containing ubiquitin conjugating enzyme (BRUCE/ Apollon/BIRC6), Survivin (TIAP/BIRC5) and Livin (KIAP/ ML-IAP/BIRC7) (Dai et al., 2009a).

The defining characteristic of the IAP family is the presence of at least one Baculovirus IAP Repeat (BIR) domain. The human IAPs contain either one (Survivin, Livin, TsIAP, BRUCE) or three (XIAP, cIAP1, cIAP2, NAIP) tandem BIR domains. Along with BIR domains, several IAPs contain a RING (really interesting new gene)-zinc finger domain at carboxy terminus. Both c-IAP1 and c-IAP2 contain a caspase recruitment domain (CARD) in the linker region between the BIR domains and the RING domain. NAIP contains a nucleotide binding site domain as well as a leucine-rich repeat domain. BRUCE contains a ubiquitin-conjugation domain (Figure 1.2). In XIAP, c-IAP1 and c-IAP2, the RING domain has been shown to possess E3 ubiquitin ligase activity directly regulating self-ubiquitination and protein degradation (Hunter et al., 2007).

IAP proteins are divided into two groups based on their binding properties to caspases and inhibition of caspase activity. The first group includes XIAP, cIAP-1, cIAP-2, Livin, NAIP and TsIAP; these IAPs bind to and inhibit caspase-3, -7 and -9 and function as potent apoptosis inhibitors. The second group includes Survivin and BRUCE; these IAPs do not bind to caspases and in addition to their anti-apoptotic activity, rather they regulate cytokinesis and mitototic spindle formation (Yang et al., 2004). IAPs are the only known endogenous proteins that regulate the activity

of both initiator and effector caspases (LaCasse et al., 2008). IAPs inhibit apoptosis by preventing the activation of procaspases and inhibiting the enzymatic activity of mature caspases (Chai et al., 2000).

1.2.3.1 X-linked inhibitor of apoptosis protein (XIAP)

XIAP is a 57 kDa protein that is expressed ubiquitously in adult and fetal tissues. XIAP is the first well characterized IAP family member due to its potent anti-apoptotic activity. XIAP is the only cellular protein that potently inhibits the enzymatic activity of mammalian caspases at both the initiation phase (caspase-9) and the execution phase (caspase-3 and -7) of apoptosis (Kashkar, 2010). Two regions in XIAP confer different specificity in the inhibition of caspase-3, -7 and -9. BIR3 domain of XIAP selectively targets caspase-9 whereas the linker region between BIR1 and BIR2 of XIAP inhibits caspase-3 and caspase-7, the effector caspases that triggers downstream apoptosis. While XIAP prevents the activation of all three caspases, it has been shown that the interaction of XIAP with caspase-9 is the most critical for its inhibition (Dai et al., 2009a). Because effector caspase activity is essential for irreversible programmed cell death, XIAP functions as a gatekeeper to this final stage of apoptosis (Li et al., 2004).

XIAP is overexpressed in many cancer cell lines and tumours compared to normal cells, and high levels of XIAP result in apoptosis-resistance of cancer cells to a wide variety of therapeutic agents (Huang et al., 2001). XIAP expression was associated with adverse tumour histology and decreased patient survival and it has been proposed as an important adverse biomarker of chemoresistance in cancer (Kashkar, 2010). However, other studies showed that elevated XIAP expression alone cannot serve as a predictive marker of chemoresistance and that in order to predict impact of XIAP on chemoresistance, both level of XIAP and the functional status of all XIAP modulators (Smac and Bcl-2) need to be taken into account (Seeger et al., 2010).

1.2.3.2 Cellular inhibitor of apoptosis proteins (cIAP-1 and cIAP-2)

cIAP-1 and cIAP-2 are weak caspase inhibitors in *vitro*. Although the role of cIAP-1 and cIAP-2 in apoptosis is less well defined than XIAP, their function on the cellular responses other than apoptosis is widely reported. cIAP-1 and cIAP-2 are associated with the TNF receptor 1 signalling complex as well as regulating NF-κB signalling (Dai et al., 2009a). cIAP-1 and cIAP-2 are identified for the ability to interact with TRAF1 and TRAF2, localizing the cIAPs to the TNF receptors. The recruitment of cIAP-1 and cIAP-2 to TNFR1 inhibits TNF-α dependent, RIPK1-dependent activation of caspase-8 (Greer et al., 2011).

1.2.4 Caspases (cysteine-aspartate proteases)

Caspases are a class of cysteine-aspartyl proteases that are synthesized as inactive precursor enzymes or proenzymes. These proteases lie dormant in the healthy cell and in response to cell death stimuli are converted, either by proteolytic cleavage or by recruitment into large complexes, into active enzymes. Once activated, caspases cleave their substrates, this is responsible for most of the biochemical and morphological features of apoptotic cell death (Hunter et al., 2007).

Caspases in their proenzyme form contain three domains, amino-terminal prodomain followed by a large (P20) subunit containing the active site cysteine and carboxy-terminal small (P10) subunits. Aspartate cleavage sites are present between prodomains and large subunit and between large and small subunits. Two cleavage events are required to activate caspases into functional proteases. The first proteolytic cleavage divides the proenzyme into large and small subunits. A second cleavage event removes the amino terminal prodomain. The resultant functional caspase is a tetramer of two large and two small subunits (Figure 1.3A).

14 mammalian caspases are known. Based on their function, the caspases can be classified into three groups as shown in Figure 1.3B:

1) Inflammatory caspases, this group includes caspase-1, -4, -5, -11, -13 and -14 which are involved in inflammation.

2) Apoptotic initiator caspases, initiator caspases have a long prodomain containing either a death effector domain (DED) (caspase-8 and -10) or a caspase activation and recruitment domain (CARD) (caspase-2 and -9).

3) Apoptotic effector caspases, which include caspase -3, -6 and -7 and they are characterised by the presence of a short prodomain. (Degterev et al., 2003).

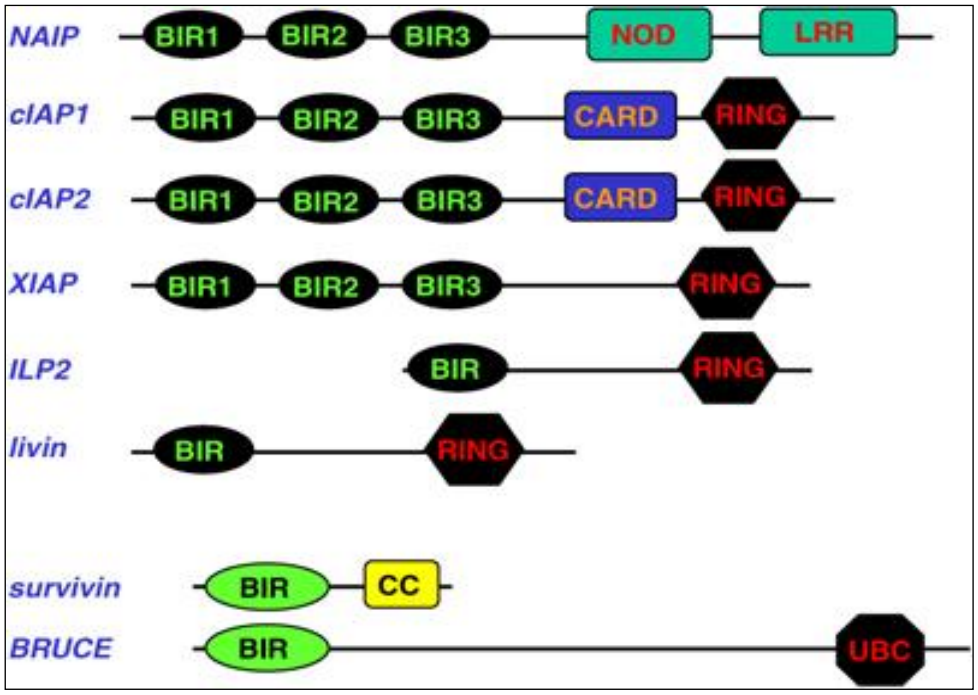
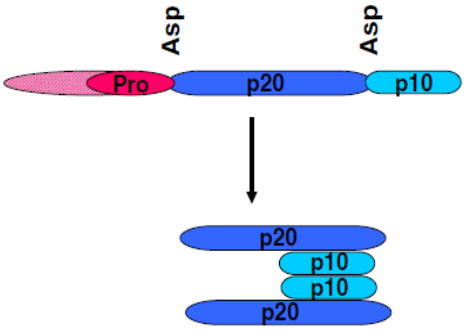


Figure 1.2 Domain structure of the mammalian IAPs

The domain organization is shown for the eight mammalian IAPs. The presence of at least one baculovirus IAP repeat (BIR) domain is the defining characteristic of the IAP family. The human IAPs possess either one (survivin, BRUCE, livin and ILP2) or three tandem amino-terminal BIR domains (XIAP, cIAP1, cIAP2 and NAIP). Several IAPs contain an E3 ubiquitin ligase zinc-finger (RING) domain at the carboxy terminus. Both cIAP1 and cIAP2 possess a caspase-recruitment (CARD) domain in the linker region between the BIR and the RING domains. Uniquely, NAIP possesses a nucleotide-binding and oligomerization (NOD) domain. BRUCE contains an ubiquitin conjugating (UBC) domain but no RING domain. Survivin contains a coiled-coil (CC) domain that binds chromosomal passenger proteins. From LaCasse et al., 2008.

A



B

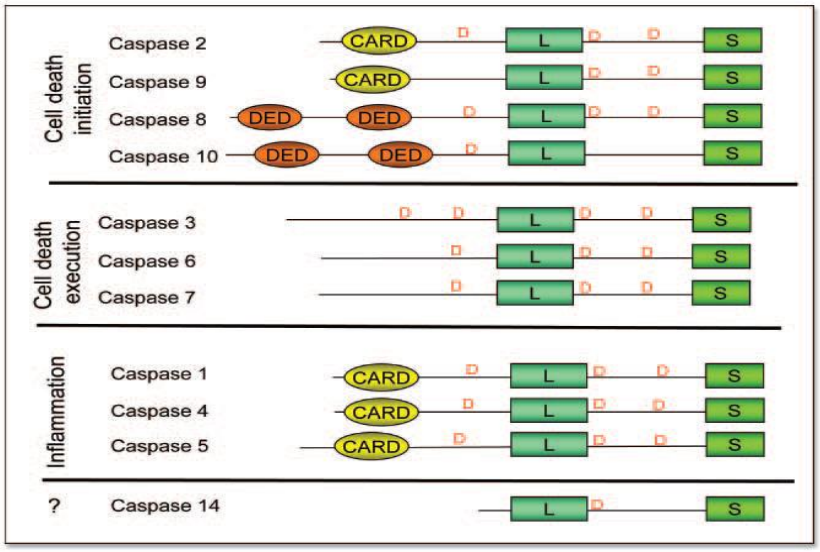


Figure 1.3 Mammalian caspases; activation, structure and classification

A: Structure of caspases as pro-enzymes and after activation, from Hunter et al., 2007; Caspases are synthesized as inactive zymogens. Cleavage at aspartate (Asp) sites results in an active tetramer consisting of two large (p20) and two small (p10) subunits. B: Classification of mammalian caspases, from Jin and El-Deiry, 2005; Caspases can be classified into three groups: 1) Inflammatory caspases, include caspase-1, -4, -5, -11, -13 and -14. 2) Apoptotic initiator caspases, initiator caspases posses long prodomain containing either a death effector domain (DED) (caspase-8 and -10) or a caspase activation and recruitment domain (CARD) (caspase-2 and -9). 3) Apoptotic effector caspases, which include caspase -3, -6 and -7 and they are characterized by the presence of a short prodomain. Orange D letter indicates aspartate cleavage sites.

1.2.5 Cellular FLICE inhibitory protein (cFLIP)

cFLIP is a cellular protein that shares high sequence homology with the initiator caspases. It inhibits caspase activation at the death-inducing signalling complex (DISC) by competing for binding to the death effector domain (DED) of Fas associated death domain protein (FADD). cFLIP exists as many splice variants, of which only a longer ($cFLIP_L$) and a shorter version ($cFLIP_S$) could initially be detected at the protein level. Both variants contain DED domains that are structurally similar to the N-terminal part of pro-caspase-8. The C-terminus of $cFLIP_L$ consists of two catalytically inactive caspase-like domain (P20 and P10), whereas the short C-terminus of $cFLIP_S$ does not (Newsom-Davis et al., 2009).

1.2.6 Bcl-2 family proteins

Bcl-2 family proteins are group of proteins involved in cell death/survival balance. At least 20 Bcl-2 family members have been identified in mammals. The key determinant of a Bcl-2 related protein is the presence of at least one of four conserved Bcl-2 homology (BH1-BH4) domains. The Bcl-2 family proteins are divided into three groups according to their structure and function (Figure 1.4):

1) Anti-apoptotic, usually contain all four BH domains and includes Bcl-2, Bcl-X_L, Bcl-W, Mcl-1 and A1/Bfl-1.

2) Multi-domain pro-apoptotic, containing BH1, BH2 and BH3 and include Bax, Bak and Bok/Mtd.

3) BH3-only proteins, which contain only BH3 domain and include Bid, Bim/Bod, Bad, Bmf, Bik/Nbk, Bik, Noxa, Puma/Bbc3 and Hrk/DP5. BH3-only members stimulate apoptosis either by interaction with anti-apoptotic proteins to inhibit their function and/or by interaction with pro-apoptotic proteins to stimulate their activity (Martinou and Youle, 2011).

Many Bcl-2 members have a conserved C-terminal transmembrane (TM) region that mediates their localization to different organelles including the nuclear

envelope, the endoplasmic reticulum and the outer mitochondrial membrane. Bcl-2 family proteins act at the mitochondrial level, regulating the exit of apoptogenic factors such as cytochrome c that are sequestered in mitochondria (Fleischer et al., 2006).

1.2.6.1 Bcl-2 protein

Bcl-2 protein is an anti-apoptotic member of the Bcl-2 family. It is a 26 kDa protein that is localized in the mitochondria, endoplasmic reticulum and perinuclear membrane (Korsmeyer, 1995). Bcl-2 belongs to the proto-oncogene family but the ability of Bcl-2 to promote tumour growth is different from other oncogenes; it can rescue cells that otherwise destined to die without affecting their proliferation rate (Kinloch et al., 1999). Bcl-2 is involved in maintaining cellular homeostasis, including the mitochondrial membrane status and balance of the interaction between the members of the Bcl-2 protein family. In addition, Bcl-2 has a role in the regulation of calcium homeostasis in the cell (Veis et al., 1993).

Bcl-2 protein overexpression was found to mediate resistance to TRAIL induced apoptosis in neuroblastoma, glioblastoma and breast carcinoma cell lines in which Bcl-2 overexpression reduced TRAIL-induced cleavage of caspase-8 and Bid and blocked cleavage of caspases-9, -7 and -3 into active subunits (Fulda et al., 2002). However, Ndozangue-Touriguine et al. showed that there was no difference in expression level of Bcl-2 between primary colon carcinoma cell line (SW480, TRAIL sensitive) and the metastatic cell line (SW20, TRAIL resistance) (Ndozangue-Touriguine et al., 2008).

1.2.6.2 Mcl-1 protein

Mcl-1 (myeloid cell leukaemia-1) is another anti-apoptotic member of the Bcl-2 family protein originally identified in differentiating myeloid cells in 1993 (Kozopas et al., 1993). Mcl-1 is expressed in a wide variety of cell types (Krajewski et al., 1995) and Mcl-1 expression is highly induced by survival and differentiation signals such as cytokines and growth factors (Craig, 2002). Mcl-1 contains BH domains1–3, but lacks the N-terminal BH4 domain. Like many other Bcl-2 family

proteins, Mcl-1 also contains a C-terminal transmembrane (TM) domain that serves to localise Mcl-1 to various intracellular membranes, particularly outer mitochondrial membrane (Yang et al., 1995). Mcl-1 also plays a critical role in the survival of malignant cells because depletion of Mcl-1 via antisense oligodeoxynucleotides triggers apoptosis in cancer cells (Derenne et al., 2002). It is believed that Mcl-1 induces its anti-apoptotic effect via suppression of cytochrome c release from the mitochondria, possibly via heterodimerization with, and neutralisation of, pro-apoptotic Bcl-2 family proteins (Michels et al., 2005).

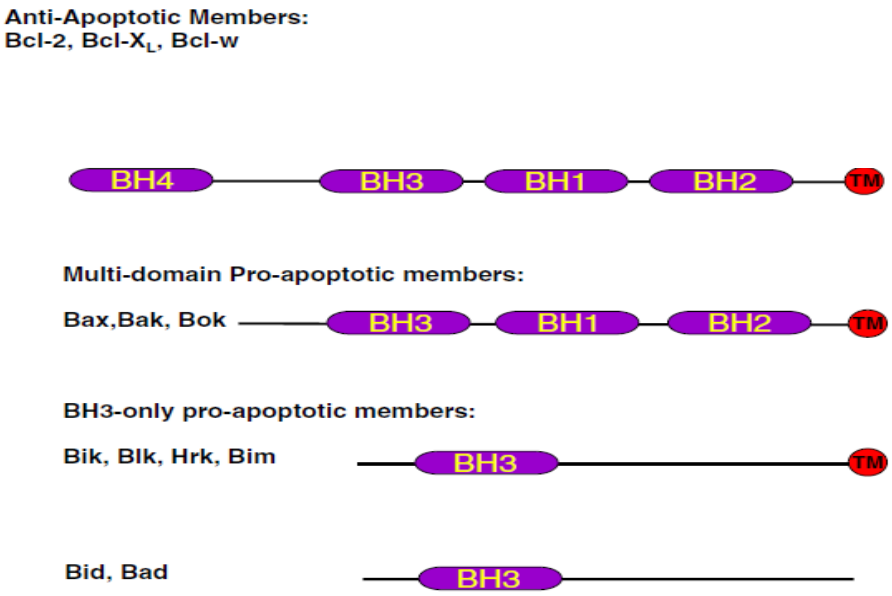


Figure 1.4 Bcl-2 family members

The Bcl-2 family members are divided into three groups of proteins according to their structure and function. The anti-apoptotic family members usually contain all four BH domains and include Bcl-2, Bcl-XL, and Bcl-w. The multi-domain pro-apoptotic members are lacking the BH4 domain and include Bax, Bak, and Bok. Finally, the BH3-only pro-apoptotic family members contain only the BH3 homology domain and include Bid, Bad, Bim, Bik, Blk, and Nox. Adapted from Hunter et al., 2007.

1.2.7 Nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB)

NF-κB is a protein complex that control transcription of DNA. It was first discovered in David Baltimore's laboratory via its interaction with an 11 base pair sequence in the immunoglobulin light chain gene in B cells (Sen and Baltimore, 1986). The NF-κB family consists of several members categorised into two classes; class I which includes NF-κB1 and NF-κB2 and class II which includes Rel1A, Rel1B and c-Rel.

The activity of NF-*κ*B is tightly regulated by interaction with inhibitory I*κ*B proteins. As with the NF-*κ*B transcription factors, there are several I*κ*B proteins, I*κ*B*α*, I*κ*B*β*, I*κ*B*γ* and I*κ*B*ε* that have different affinities for individual NF-*κ*B dimers. Individual I*κ*Bs are regulated by phosphorylation and proteolysis, and show differences in their tissue-specific expression patterns (reviewed by (Chen and Ghosh, 1999). In most cells, NF-*κ*B is present as a latent, inactive, I*κ*B-bound complex in the cytoplasm. Activation of NF-*κ*B is mediated via activation of canonical (classical) and non-canonical (alternative) pathways (Hoffmann et al., 2006).

In the canonical pathway, the binding of a ligand to a cell surface receptor recruits adaptors (e.g., TRAFs and RIP) to the cytoplasmic domain of the receptor. In turn, these adaptors often recruit an IKK (inhibitor of κB kinase complex) complex leading to activation of the IKK complex. IKK then phosphorylates I*κ*B which leads to its ubiquitination and degradation by the proteasome. NF-*κ*B then enters the nucleus to activate target genes. The non-canonical pathway is for activation of p100/RelB complexes during B- and T-cell organ development. n the non-canonical pathway, receptor binding leads to activation of the NF-*κ*B-inducing kinase NIK, which phosphorylates and activates an IKK*α* complex, which in turn phosphorylates p100, leading to its partial proteolysis and liberation of the p52/RelB complex (Gilmore, 2006).

1.2.8 Tumour necrosis factor-related apoptosis inducing ligand (TRAIL)

Tumour necrosis factor belongs to a family of cytokines that interact with a corresponding set of receptors of the TNF receptor family. Signals induced by these interactions serve diverse functions including differentiation, proliferation, activation or induction of cell death. Two of these ligands CD95L and TNF are able to induce apoptosis in transformed cells but they have acute toxic effects on normal tissues which limit their use as anticancer agents (Cosman, 1994). TRAIL was first identified by Wiley and co-workers in 1995 as a 281 amino acid type II transmembrane protein, whose C-terminal extracellular domain showed clear homology to other TNF family members and its transcripts are detected in a variety of human tissues, mostly in spleen, lung and prostate (Wiley et al., 1995).

Early studies identified two unique characteristics of TRAIL upon other members of TNF family (FasL and TNF). First, TRAIL induced apoptosis occurs only in tumourigenic or transformed cells and not normal cells (Wiley et al., 1995). Second, mRNA for TRAIL is detected in a wide range of tissues (Kayagaki et al., 1999). It has been suggested that TRAIL's selectivity is in part due to the presence of decoy receptors on normal cells that prevent TRAIL from binding to death receptors and inhibits TRAIL-induced apoptosis (Kimberley and Screaton, 2004).

1.2.8.1 TRAIL receptors

Five different TRAIL receptors have been identified. Two are death receptors, TRAIL-R1 (death receptor DR4) and TRAIL-R2 (death receptor DR5) which are able to transmit an apoptotic signal. They contain a death domain (DD) in their intracellular portion, a six to seven α-helices motif which can bind to other DDs via homotypic interaction. There are two decoy receptors, TRAIL-R3 (decoy receptor DcR1) which lacks DD and TRAIL-R4 (decoy receptor DcR2) with a truncated death domain (DD) that could induce activation of NF-κB. TRAIL also binds a soluble receptor called osteoprotegerin (OPG) which is involved in the regulation of bone formation (Newsom-Davis et al., 2009).

1.2.8.2 Recombinant human TRAIL

Early studies showed that full length cell surface expressed TRAIL and soluble TRAIL induced apoptosis in a wide variety of transformed cell lines (Wiley et al., 1995). This discovery has been followed by production of several forms of recombinant TRAIL. In 1999, Ashkenazi and co-workers generated a soluble version of the native Apo2-L (TRAIL) protein and showed that the recombinant protein induced cytostatic or cytotoxic effect on 32 of 39 cell lines from colon, lung, breast, kidney, brain and skin cancer in vitro. It also reduced tumour incidence and tumour progression in a mouse model without affecting normal cells or tissues (Ashkenazi et al., 1999). Walczak and co-workers also created leucine Zipper forms of human TRAIL which was able to induce apoptosis in target cells in *vitro* and was able to reduce tumour growth in SCID (Severe Combined Immunodeficiency) mice with no toxicity to normal tissues (Walczak et al., 1999). Consequently TRAIL has emerged as a promising targeted cancer therapy.

A variety of different forms of human recombinant soluble TRAIL have been designed, each encoding the extracellular domain of human TRAIL. Some preparations are amino-terminally fused to either polypeptide tags (Pitti et al., 1996), FLAG epitope (Schneider, 2000), leucine Zipper (LZ) (Ganten et al., 2006; Walczak et al., 1999) or isoleucine Zipper (IZ) trimerization domains (Ganten et al., 2006).

To overcome the short half life and toxicity of high doses of soluble TRAIL, another method of introducing TRAIL to tumour tissues was developed in mouse model. Adenovirus infected $CD34^+$ cells expressing membrane bound TRAIL have been generated and tested using a subcutaneous myeloma model in immunodeficient mice via intravenous injection. The transduced cells were found to home in the tumour peaking at 48 hours. Both CD34-TRAIL expressing cells and soluble TRAIL significantly reduced tumour volume by 40% and 29%, respectively. CD34-TRAIL expressing cells but not soluble TRAIL significantly damaged tumour vasculature suggesting that tumour homing of CD34-TRAIL expressing

cells induces early vascular disruption, resulting in hemorrhagic necrosis and tumour destruction (Lavazza et al., 2010).

Some recombinant TRAIL variants may induce systemic toxicity; polyhistidine tagged TRAIL has been shown to induce apoptosis in normal human hepatocytes (Jo et al., 2000) and recombinant human leucine zipper (LZ) and polyhistidine tagged have been shown to induce apoptosis in normal keratinocytes (Leverkus et al., 2000). Recombinant LZ-TRAIL was cytotoxic to astrocytes in vitro (Walczak et al., 1999).

Conversely, recombinant TRAIL that lacked exogenous sequences did not induce apoptosis in normal cynomolgus monkey hepatocytes (Lawrence et al., 2001), human mammary, renal or prostatic epithelial cells, umbilical vein endothelial cells, lung fibroblasts, colon smooth muscle cells, astrocytes or keratinocytes (Ashkenazi et al., 1999; Qin et al., 2001) and thus has been selected as the most promising candidate for clinical trials in cancer patients. Based on these results, a phase I study with rhTRAIL (Genentech, San Fransisco, CA, USA) has been initiated (Duiker et al., 2006). The results from phase I clinical trials showed that 50% of 32 evaluable patients with solid and haematological malignancies achieved stable disease and one patient demonstrated a partial response in doses up to 15 mg/kg indicating that Apo2L/TRAIL was safe and well tolerated when used in escalating doses for patients with advanced cancer in phase I trials (Herbst et al., 2010).

1.2.8.3 TRAIL-mediated apoptosis

TRAIL induces cell death by activation of apoptosis pathways following binding to its corresponding receptors. After binding of TRAIL to TRAIL-R1 or R2, the trimerized receptors recruit several cytosolic proteins that form the death-inducing signaling complex (DISC). FADD binds directly to the intracellular death domain of the receptors where it binds the inactive pro-form of caspase-8 or caspase-10 leading to their activation. Then two different pathways can be triggered, one whereby caspase-8 directly activates the effector caspases (caspase-

3,-6,-7) leading to cell death, and the other involving caspase-8 dependent cleavage of Bid thus engaging the intrinsic death pathway. Cleaved Bid translocates to the mitochondria where it interacts with Bax and Bak to form pores in the mitochondrial membrane, leading to the release of cytochrome c and Smac/DIABLO from the mitochondria. Cytochrome c together with Apaf-1 and procaspase-9 form the apoptosome which leads to caspase-9 activation and subsequent activation of effector caspases (Kruyt, 2008).

Recent studies demonstrated that TRAIL can sometimes promote survival, proliferation, migration and invasion in TRAIL-resistant tumour cells. Binding of TRAIL to death receptors (DR4 and DR5) and even to decoy receptor (DecR2) induces NF-κB activation (Degli-Esposti et al., 1997). TRAIL-induced NF-κB activation was initially suggested to be a mechanism for negative regulation of TRAIL-induced apoptosis. However, in TRAIL-resistant cells, it has been shown to mediate RIP-dependent TRAIL-induced survival and proliferation in Jurkat cells (Ehrhardt et al., 2003). In apoptosis-resistant cholangiocarcinoma cells, TRAIL has been shown to promote NF-κB-dependent tumour cell migration and invasion, but not proliferation (Ishimura et al., 2006).

1.2.8.4 TRAIL resistance in primary tumours

The majority of human primary tumour cells are resistant to TRAIL-mediated apoptosis. However, treatment with chemotherapeutic or other biological agents can sensitise many primary tumour cells to killing by TRAIL while leaving normal cells largely unaffected. The mechanisms of TRAIL resistance has been extensively studied and several mechanisms have been proposed; overexpression of DcR1 and DcR2 by acting as decoys and competing against DR4 and DR5 (LeBlanc and Ashkenazi, 2003), elevated cFLIP expression (Clarke and Tyler, 2007), high expression of IAPs (Makhov et al., 2008) and overexpression of Bcl-2 (Fulda et al., 2002) and Bcl-xl (Hinz et al., 2000). In colon carcinoma cells, TRAIL resistance has been proposed to be due to overexpression of XIAP and mitochondrial block but it was not related to Bcl-2, Bcl-xL and Mcl-1 levels (Ndozangue-Touriguine et al., 2008).

A novel mechanism of TRAIL resistance in glioblastoma cells was found to be due to overexpression of ubiquitin-modifying enzyme A20. After TRAIL receptor stimulation, A20 mediates the polyubiquitination of RIP1 at the TRAIL receptor tail, resulting in the interaction of polyubiquitin chain to procaspase-8 that is recruited to the TRAIL-bound receptors. The inability of ubiquitin-bound procaspase-8 to be dimerized and activated prevents the execution of the apoptosis (Bellail et al., 2012; Verbrugge and Johnstone, 2012).

1.2.8.5 TRAIL sensitisation

Although some tumour cells develop resistance to TRAIL, there are various sensitising agents that could mediate the reversal of TRAIL resistance. Nitrous oxide was used to sensitise TRAIL resistant cells by its inhibitory effect on NF-κB and upregulation of TRAIL death receptor 2 (DR5) (Lee et al., 2007). Proteasome inhibitor MG132 sensitised hepatocellular carcinoma to TRAIL via inhibition of the NF-κB pathway (Ganten et al., 2005). Similarly, proteasome inhibitor Bortezomib has been used to sensitise malignant human glioma cells (Jane et al., 2011).

Ultraviolet radiation has been used to sensitise melanoma cells to TRAIL-mediated apoptosis. The sensitisation was due to XIAP degradation, which allowed full processing of caspase-3. It was also accompanied by degradation of IκBα (Inhibitor of κB), resulting in NF-κB-dependant transcriptional repression of XIAP (Thayaparasingham et al., 2009). Neuroblastoma cells were sensitised to TRAIL-mediated apoptosis by inducing NF-κB inhibition using IκBα-superrepressor or small molecule NF-κB inhibitor BMS-345541. The sensitisation was because NF-κB inhibition prevented TRAIL-triggered up-regulation of Mcl-1 promoting TRAIL-induced cytochrome c release and caspase activation (Ammann et al., 2009). Smac mimetics were used to sensitise breast cancer cells to TRAIL through their binding to IAPs and consequent caspase-3 activation (Fandy et al., 2008; Li et al., 2004) and use of XIAP inhibiting compound was able to sensitise chronic lymphocytic leukaemia cells to TRAIL-mediated apoptosis (Frenzel et al., 2011).

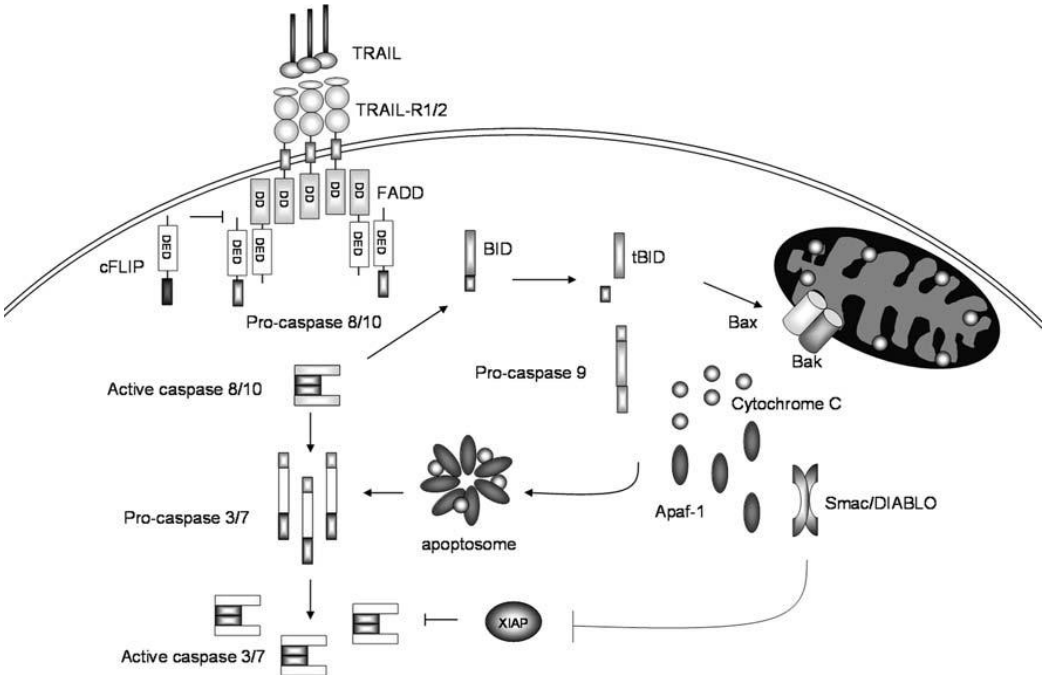


Figure 1.5 Model of TRAIL mediated apoptotic signalling

Upon binding of trimeric TRAIL to TRAIL-R1/-R2, FADD associates with the receptors' death domains (DD) enabling the recruitment of caspase-8/10 and cFLIP to the DISC. In the direct apoptotic pathway, activated caspase-8/10 cleaves downstream effector caspases. Type II cells require pro-apoptotic changes at the mitochondria (the intrinsic pathway) resulting in apoptosome formation and release of Smac/DIABLO. From Newsom-Davis et al., 2009.

1.2.9 Second mitochondria-derived activator of caspases, direct IAP-binding protein with low PI (Smac/DIABLO)

Smac/DIABLO (second mitochondria-derived activator of caspases, direct IAP-binding protein with low PI) is a 25-kDa mitochondrial protein. The discovery of Smac was first published in 2000 by Du et al. and at the same time by Verhagen et al. who named the protein DIABLO, hence the name Smac/DIABLO (Du et al., 2000; Verhagen et al., 2000). Smac resides within the mitochondrial intermembrane space and released into cytosol upon the induction of apoptosis (Chen and Huerta, 2009).

Smac/DIABLO promotes apoptosis through its ability to antagonize IAP-mediated caspase inhibition once released into the cytoplasm. Initial mitochondrial targeting of Smac depends on 53-55 amino acid sequence found at its amino terminus. This sequence is proteolytically cleaved within the mitochondria to generate AVPI (Ala-Val-Pro-Ile) containing amino terminus polypeptide, which is sequestered until an apoptotic stimulus is sensed. On receiving an apoptotic signal through the intrinsic mitochondrial pathway, both Smac and cytochrome c are released through mitochondrial membrane pores (LaCasse et al., 2008).

1.2.9.1 Function of Smac/DIABLO

Smac/DIABLO is considered a general IAP inhibitor due to its ability to bind to XIAP, c-IAP1, c-IAP2, Survivin, Livin and BRUCE but not NAIP. Smac is able to interact with IAPs by binding to BIR2 and BIR3 domains but not BIR1 domain. The first four amino-terminal residues of mature Smac/DIABLO, AVPI are required for Smac/DIABLO function. Activation of Smac/DIABLO is mediated by homodimerization to form a stable protein dimer in which the amino terminus AVPI makes contact with XIAP BIR3. This prevents XIAP from binding to caspases, thus freeing caspases from inhibition (LaCasse et al., 2008).

In HNSCC cell lines, Smac mediates apoptosis induced by several chemotherapeutic agents through the mitochondrial pathway and Smac knockdown led to impaired caspase activation, mitochondrial depolarization and cytochrome c

release. Furthermore, Smac levels modulated the therapeutic response of HNSCC cells to gemicitabine in xenograft models (Sun et al., 2011). Smac/DIABLO expression was lower in oesophageal squamous cell carcinoma tissue than normal counterpart. Furthermore, the level of Smac expression tested by immunohistochemistry using tissue microarray correlated with the response to chemotherapy (high Smac, better response) and Smac downregulation induced chemoresistance to cisplatin and inhibited caspase3 activation. Furthermore, Smac mimetics restored the sensitivity of oesophageal squamous cell carcinoma to cisplatin. (Xu et al., 2011).

1.2.9.2 Smac isoforms

There are two additional Smac isoforms Smacβ and Smac3. Smacβ is an alternatively spliced cytoplasmic isoform of Smac that lacks the mitochondrial targeting sequence. Smacβ is still considered to be proapoptotic due to its ability to potentiate apoptosis following death receptor activation and chemical stimuli. Smac3 isoform contains both mitochondrial targeting and IAP binding motif sequence. Similar to Smac, upon apoptotic stress, Smac3 is released from the mitochondria into the cytoplasm where it interacts with XIAP. Smac3 is able to induce the acceleration of XIAP auto-ubiquitination and degradation whereas Smacβ only has this effect on cIAP1 and cIAP2 (LaCasse et al., 2008).

1.2.9.3 Smac mimetics

Smac/DIABLO is the natural inhibitor of XIAP activity that is released from the mitochondria to the cytoplasm following activation of the intrinsic pathway. Smac/DIABLO removes the inhibitory effect of XIAP on effector caspases allowing for apoptosis execution. Smac mimetics are molecules that echo the action of Smac/DIABLO and different types of Smac mimetics have been introduced as potential anticancer agents in multiple cancers; these include Smac based peptides, polynucleotides and small drug compounds.

1.2.9.3.1 Peptides mimetics

A Fusion peptide of the last four to eight N-terminal residues of Smac combined with a carrier protein (Drosophila antennapaedia penetratin sequence) was able to enhance apoptosis induced by chemotherapeutic drugs (paclitaxel, etoposide, 7-ethyl-10-hydroxycamptothecin (SN-38), and doxorubicin) in MCF-7 and other breast cancer cell lines. It has been shown that the Smac-penetratin fusion peptide crossed the cell membrane, bound XIAP and cIAP1, displaced caspase-3 from cytoplasmic aggregates, and enhanced drug-induced caspase activation (Arnt et al., 2002).

1.2.9.3.2 Polynucleotides mimetics

Plasmid DNA containing full length and mature Smac increased sensitivity of leukaemia cell lines to UV (ultraviolet radiation) and TRAIL induced apoptosis (Jia et al., 2003).

1.2.9.3.3 Small molecules mimetics

Compound 3 was the first reported small molecule mimetic in 2004 by Li et al. (Li et al., 2004). Since then, many other Smac mimetic molecules have been introduced including SM-164 also known as Smac59 which is a bivalent Smac mimetic that has cell-permeable capabilities (Sun et al., 2007), Low molecular weight LBW242 Smac mimetics (Weisberg et al., 2007), Compound A (Vince et al., 2007) and Smac37 (Cossu et al., 2009).

1.2.9.4 Smac mimetics- induced apoptosis

Several mechanisms by which Smac mimetics could induce apoptosis have been proposed; induction of TNF-α dependent apoptosis (Petersen et al., 2007), blocking of IAPs (Bockbrader et al., 2005), or antagonizing caspase-IAP interactions and repressing IAP ubiquitin ligase activities (Creagh et al., 2004). Recently, it has been proposed that Smac mimetics induce apoptosis via formation of a cellular complex called ripoptosome which contains receptor interacting protein

1 (RIP1), FADD and caspase-8 and it assembles in response to Smac-mimetic induced depletion of XIAP, cIAP1 and cIAP2 (Tenev et al., 2011).

Smac mimetics have been used successfully as a sensitiser for other therapeutics; compound 3 potentiated TRAIL and TNF-α-mediated cell death in human glioblastoma cells (Li et al., 2004), SH122 Smac mimetics sensitized prostate cancer cells to TRAIL-induced apoptosis (Dai et al., 2009b) and Smac mimetic JP1201 sensitised non-small cell lung carcinoma cells to different standard chemotherapeutic drugs (Greer et al., 2011). Probst et al. showed that the combination effect of Smac mimetics and conventional chemotherapy in variable cancer cell lines was because of multiple mechanisms involving both inhibition of cell proliferation by the chemotherapy agents and enhanced autocrine TNF-α feedback loop by the Smac mimetic/chemotherapy combination (Probst et al., 2010).

1.2.10 TNF-alpha

TNF-α is a cytokine that is involved in many biological responses, including inflammation, cell proliferation, differentiation and apoptosis. Although the molecular mechanisms of TNF signaling have been largely elucidated, the principle that regulates the balance between life and death is still unknown (Dai et al., 2009a).

Several potential mechanisms for the involvement of TNFα signalling in Smac mimetic-induced apoptosis in cancer cells have been proposed. Smac-mimetics stimulate autoubiquitination of cIAPs, resulting in their proteasomal degradation. This is in turn leads to NIK stabilization and facilitates RIP1 recruitment and results in the activation of non-canonical and canonical NF-κB pathways, causing autocrine TNF-α production in many cell lines (Lu et al., 2007). Blocking NF-κB activation reduced TNF-α production and protected cells from Smac mimetic induced cell death (Ahn et al., 2007).

Autocrine TNF-α secretion was a strong predictor of sensitivity to JP1201 Smac mimetics in non small cell lung carcinoma cells as the sensitive cells showed higher levels of TNF-α in their growth media. However, the sensitisation induced

by JP1201 in resistant cells to Vinorelbine was independent of TNF-α because knockdown of RIPK1, caspase8 or TNFR1 did not protect cells from combined treatment with JP and Vinorelbine (Greer et al., 2011). Autocrine TNF-α contributes to Smac mimetic-induced tumour regression as a single agent or in combination with chemotherapeutics in HCC461 lung cancer cell xenograft mouse models (Probst et al., 2010).

1.3 DNA damage repair

The initial reaction of a cell to DNA damage is to repair the damage. However, with increasing DNA damage the cell triggers cell cycle arrest or apoptosis. The maintenance of this switching mechanism is important for avoiding progression to cancer (Bernstein et al., 2002). DNA damage repair capacity varies between individuals as a result of inheritance, environmental factors and physiological factors. Cytogenetic and molecular analysis in oral cancer have shown changes in several chromosomes and genes, including those involved in the repair of DNA damage (Scully et al., 2000).

Five major DNA repair pathways exist. In each of these DNA repair pathways, different proteins function to detect DNA damage and repair (Bernstein et al., 2002):

1. Homologous recombinational repair (HRR)

In homologous recombinational repair sequence information that is lost due to damage in one double stranded DNA molecule is accurately replaced by physical exchange of a segment from the homologous intact DNA molecule.

2. Non homologous end joining (NHEJ)

Non homologous end joining repairs double strand breaks and involves re-joining of the broken ends.

3. Nucleotide excision repair (NER)

Nucleotide excision repair repairs DNA with double helix-distorting damage caused by ultraviolet light or chemotherapeutic agents. It involves recognition of

DNA defects, incision and removal of damaged strands and filling the gap by repair synthesis.

4. Base excision repair (BER)

Base excision repair is a major DNA repair pathway protecting against single-base DNA damage caused by methylating and oxidizing agents.

5. Mismatch repair (MMR)

Mismatch repair is responsible for the post-replication correction of nucleotide mispairs and extrahelical loops as well as repairing DNA damage caused by variety of DNA damaging agents.

Studying the DNA damage responses is important for understanding cancer biology as DNA damage causes cancer, affects response to treatment and contributes to the side effects of treating cancer (Kastan, 2008). Impaired DNA repair was correlated with sensitivity of HNSCC cells to γ radiation suggesting that DNA repair might be critical for HNSCC treatment (Rusin et al., 2009).

1.3.1 Excision repair cross complementation group 1 (ERCC1)

Excision repair cross complementation group 1 (ERCC1) is a protein component of the nucleotide excision repair complex. It is involved in both recognition of the damage and excision of the damaged part of DNA (Hoeijmakers, 2001). Expression of ERCC1 was correlated with resistance of HNSCC patients to cisplatin-based chemotherapy (Handra-Luca et al., 2007). Genetic polymorphism in ERCC1 was found to be correlated to the response to radiotherapy in early head and neck cancers (Carles et al., 2006). In non small lung carcinoma, patients with cancers that were immunopositive to ERCC1 expression survived longer than patients with ERCC1 negative cancers and multivariate analysis suggested that ERCC1 expression was an independent prognostic marker for long survival (Lee et al., 2008). ERCC1 may be an important prognostic marker and the correlation between ERCC1 expression and the clinical features or the survival of the patients with HNSCC treated by radiotherapy remains to be investigated.

1.3.2 Hypoxia inducible factor -1alpha (HIF-1α)

Human cancers frequently contain areas of necrosis in which cancer cells have died due to inadequate oxygenation (hypoxia). A major mechanism mediating adaptive responses to hypoxia is the regulation of transcription by HIF-1. HIF-1 is a heterodimeric transcription factor composed of constitutively expressed HIF-1β subunit and O_2-dependaent HIF-1α subunit. Under normoxic conditions HIF-1α degrades rapidly but under hypoxic conditions, HIF-1α accumulates in the nucleus, dimerizes with HIF-1β subunit and activates transcription of more than 100 target genes that are responsible for inducing cell proliferation, angiogenesis, local invasion and metastasis. Increased levels of HIF-1α in tumours have been associated with poor clinical outcome in different tumour types for example breast, cervical and endometrial cancers (Semenza, 2010). Hypoxia influences the response of HNSCC to radiotherapy. Radiation acts by generating highly reactive oxygen free radicals that damage DNA and this process is enhanced in the presence of oxygen. Hypoxia inducible factor-1 α (HIF-1α) is a key factor in hypoxia and increased HIF-1α expression in HNSCC has been correlated with poor loco-regional control when radiotherapy is the primary treatment (Silva et al., 2007).

HIF-1α expression was a more significant adverse prognostic factor in the tonsil than the tongue base tumours and was significantly correlated with loco-regional control and cancer specific survival (Silva et al., 2008). Low HIF-1α expression was significantly related to disease free survival in T2 stage oral tongue cancer patients (Roh et al., 2009). HIF-1α inhibitors sensitised tumours to radiation therapy (Semenza, 2010). However, HIF-1α expression was not predictive for radio-sensitivity in human lung cancer cell lines (Schilling et al., 2012). Whether HIF-1α level could be used as a prognostic predictor for HNSCC patient treated with radiotherapy remains unclear.

1.3.3 Ku80

Ku is a nuclear protein originally identified as an autoantigen that was recognized in the sera of a Japanese patient with scleroderma polymyositis overlap

syndrome. The name Ku was derived from the first two letters of the family name of the patient. Ku protein is a complex of two subunits, Ku70 and Ku80. Ku protein plays a key role in multiple nuclear processes, for example, DNA repair, chromosome maintenance and transcription regulation. Ku protein is important for initiation of non homologous DNA end joining repair, for which heterodimerization between Ku70 and Ku80 is essential. Cells genetically deficient in either Ku70 or Ku80 are particularly sensitive to ionizing radiation (Koike, 2002).

Ku80 overexpression is a common feature of HNSCC and highly predictive of loco-regional failure and death (Moeller et al., 2011). In vitro studies have shown that Ku80 overexpression correlated with radiation resistance in HNSCC cell lines (Chang et al., 2006) and that silencing Ku80 using small interfering RNA enhanced radiation sensitivity in lung, pancreas, oesophageal, prostate and breast carcinoma cell lines (Nimura et al., 2007). These findings suggest that Ku80 expression may provide a predictive test of radiosensitivity in head and neck cancers.

1.3.4 Phosphate and tensin homolog deleted on chromosome 10 (PTEN)

PTEN was first identified as a candidate tumour suppressor gene mutated in brain, breast and prostate tumours that mapped to chromosome 10q23. It is a central negative regulator of the PI3K/AKT signalling cascades that influences multiple cellular functions including cell growth, survival, proliferation and migration. PTEN was considered to be a strictly cytoplasmic protein however, recent studies have shown that the protein localizes to the nucleus of a wide variety of tissue types and model organisms (Chow and Baker, 2006). Cytoplasmic PTEN expression was correlated with histological grade and biological behaviour of oral squamous cell carcinoma; PTEN immuno-expression was higher in well differentiated carcinomas, reduced in less differentiated tumours and was completely absent at the invasive front (Squarize et al., 2002).

In various human cancers, PTEN is frequently found to be mutated, deleted or epigenetically silenced. Recently PTEN was also found to play a critical role in DNA damage repair and DNA damage response. Cells deficient in PTEN have

defective double strand break repair (Ming and He, 2012). PTEN expression seems to plays a role in the response of cancers to radiotherapy as reduced levels of PTEN were associated with radio resistance (Ming and He, 2012) and patients with high PTEN expression had a favourable outcome after accelerated postoperative radiotherapy, compared to patients with low PTEN expression. Furthermore, adenoviral-mediated expression of PTEN sensitised lung cancer cells to radiotherapy by suppressing DNA repair capacity (Pappas et al., 2007). PTEN may serve as a valuable prognostic and predictive marker in postoperative radiotherapy for high risk HNSCC (Snietura et al., 2012).

1.3.5 Rad51

Rad51 has a central role in homologous recombinational repair. It binds to DNA and promotes homologous pairing and strand exchange, which is a central reaction in the repair process. Overexpression of Rad51 protein increased resistance of mammalian cells to ionizing radiation (Vispe et al., 1998). Similarly, antisense inhibition of Rad51 in mouse models enhanced their radio-sensitivity and Rad51 homozygous mutant mouse embryos were hypersensitive to ionizing radiation. This sensitivity was due to the loss of ability to repair double strands breaks (Bernstein et al., 2002). High levels of Rad51 protein expression have been reported in a number human cancer cell lines (Raderschall et al., 2002). Head and neck cancer patients with high Rad51 protein levels in their pre-treatment tumour biopsies demonstrated poor cancer-specific survival rates than patients with lower Rad51 levels (Connell et al., 2006). Similarly, elevated expression of Rad51 was associated with poor prognosis in oesophageal squamous cell carcinoma (Li et al., 2011).

1.3.6 X-ray repair cross complementing 1 (XRCC1)

XRCC1 protein plays an important role in repair of three common forms of DNA damage induced by ionizing radiation; single strand break repair, double strand break repair and base excision repair. After excision of damaged base, XRCC1 binds directly to the site of breaks in DNA and acts as a scaffold in the

subsequent restoration of the site. XRCC1 deficient cells show reduced rate of repair of radiation induced single strand breaks (Thacker and Zdzienicka, 2003).

High levels of ERCC1 and XRCC1 mRNA were detected in the peripheral blood of non-small cell lung carcinoma and HNSCC patients proportional to tumour expression which could allow the introduction of a simple clinical test to determine prognosis (Schena et al., 2012). High XRCC1 protein expression in HNSCC was associated with poor survival, especially in patients receiving chemoradiation (Ang et al., 2011).

1.3.7 Human papilloma virus (HPV)

HPV is a circular double-stranded DNA virus consisting of an 8-kb genome encoding viral and capsid proteins (de Villiers et al., 2004). Over 120 types have been identified and are referred to by number, classified as either high risk or low risk types on the basis of their associations with genital and other carcinomas. The common high risk types such as HPV-16 and 18 are associated with dysplasia that can progress to higher grade and to carcinoma while low risk types such as HPV-6 and 11 are associated with benign genital lesions that rarely progress to cancer (Schlecht et al., 2001).

High risk types code for at least three proteins with growth stimulating and transforming properties (E5, E6 and E7). E5 protein contributes to cellular transformation by increasing the mitogenic stimulus from growth factor receptors to the nucleus (Straight et al., 1993). The E6 and E7 proteins stimulate cellular progression through G1/S transition despite the presence of various G1 arrest signals in their host cells. E6 targets P53 protein through binding and inducing degradation. E7 targets retinoblastoma (pRb) gene causing its silencing. E2 encodes a regulatory protein which is involved in the negative regulation of E6 and E7 expression. In high risk HPV types, the integration of the viral genome into host chromosomes results in the loss of the viral E2 which leads to constitutively high E6/E7 expression levels (Lagunas-Martinez et al., 2010) and is thought to predispose to malignant transformation compared to episomal viral DNA.

HPV infection is recognized to play a role in the pathogenesis of a subgroup of HNSCC affecting tonsil and pharyngeal tissues. This subgroup is clinically characterized by the presence of high-risk HPV genomic DNA sequences in the tumours (approximately 95% contain HPV-16 DNA). Patients with HPV-positive HNSCC have a better prognosis than patients with HPV-negative HNSCC, in part because of their better therapeutic response to chemoradiotherapy (Gillison et al., 2008). HPV positive cancers probably have a different molecular and phenotypic profile from HPV negative cancers, including poor differentiation (though how to assess differentiation is contentious as the normal epithelia are non-keratinising), scant keratinization and basaloid phenotype, compared with the typically keratinizing morphology of HPV negative tumours (Miller et al., 2012).

Methods of determining HPV status in patient specimens involve detecting high risk HPV DNA or RNA either by in situ hybridization (ISH) or polymerase chain reaction (PCR). Additionally, mRNA expression of high risk HPV oncoproteins, such as E6 and E7 can be detected by qRT-PCR and indicates active infection. The p16 protein expression determined by immunohistochemistry is only an indirect marker for HPV infection (Howard and Chung, 2012) but it does indicate biologically significant high risk infection because p16 levels are only raised when E6/7 is present.

1.3.8 Cancer stem cells

Recent data indicate that the initiation, growth, recurrence and metastasis of cancers are related to the behaviour of a small population of malignant cells with properties of stem cells (Mannelli and Gallo, 2012). A cancer stem cell is defined as a cell within a tumour that possesses the capacity to self renew and to generate the heterogeneous lineages of cancer cells that comprise the tumour (Clarke et al., 2006).

Radiobiological research over the past decades has provided evidence that cancer stem cell content and the intrinsic radiosensitivity of cancer stem cells varies between tumours, thereby affecting their radio curability (Baumann et al., 2008).

Anticancer therapy can cure a tumour only if all cancer cells are killed (Dingli and Michor, 2006) and following radiotherapy any cancer stem cells left in its natural environment will cause tumour recurrence. The high proportion of cancer stem cells in tumour correlates with tumour radioresistance (Hill and Milas, 1989).

Cancer stem cells have been identified in human head and neck squamous cell carcinoma (HNSCC) using markers such as CD133 and CD44 expression, and aldehyde dehydrogenase (ALDH) activity. The head and neck cancer stem cells reside primarily in perivascular niches in the invasive front (Zhang et al., 2012). Although the existence of cancer stem cells in most solid tumours has not been formally proven, application of the cancer stem cell concept has enhanced understanding of HNSCC heterogeneity and progression (Bhaijee et al., 2012). Further researches studying differences in radioresistance between cancer stem cells and non stem cells, determination of stem cell density and distribution of stem cells within tumours is essential for future optimization of treatment strategies and for individualization of radiotherapy(Baumann et al., 2008). Therefore studying cancer stem cells in HNSCC could be helpful in understanding their response to various therapeutic modalities.

1.4 Aims of the current study

Most approaches used in cancer treatment, such as chemotherapy and radiation therapy, kill cancer cells by inducing apoptosis indirectly through the chemical or physical damage of DNA. However, cancer cells often develop resistance. Treatments that directly target and specifically activate apoptosis would be predicted to be both safer and more effective than existing therapies (Hunter et al., 2007).

This study sought to investigate prognostic and predictive markers for outcomes of HNSCC to conventional and targeted therapy as well as identifying molecular markers useful for determining preoperative prognosis through routine tumour biopsy. The overall aim of this study was to identify a genetic signature of HNSCC patients to predict response to novel targeted therapeutics as well as radiotherapy.

Specific objectives were to:

- Test the sensitivity of HNSCC cell lines to two novel targeted therapeutic agents (IZ-TRAIL and Smac mimetic, (Smac59)
- Identify biomarkers which could predict response of HNSCC cell lines to TRAIL and Smac59.
- Investigate the underlying mechanisms of TRAIL and Smac59-mediated apoptosis in HNSCC cell lines.
- Examine the expression level of different proteins involved in DNA damage repair and tumour hypoxia (ERCC1, HIF-1α, Ku80, PTEN, Rad51 and XRCC1) in HNSCC tissues using immunohistochemistry.
- Correlate expression levels of the above proteins to overall and disease free survival of the patients.
- Correlate expression levels of these proteins with the treatment outcome in the HNSCC patient with a particular emphasis on radiotherapy.

- In the present studies, in which the aim was to assess carcinoma radiosensitivity and outcome and related biomarkers, it was important to identify HPV carcinomas because they are radiosensitive, have better survival and could act as a confounding factor in analysis. These studies were not primarily targeted at HPV-associated carcinomas, which have become of increasing interest since this work started. At the outset, large numbers of HPV carcinomas were not available for analysis and only small numbers were likely to have been included in the study. The object of these experiments was to identify any HPV-associated carcinomas in the study, analyse them separately and check that they did not confound analysis of the main non-HPV carcinomas. It was not expected that there would be sufficient HPV-associated carcinomas for meaningful analysis of this subtype.

2 Materials and Methods

2.1 Materials

Plastics used in tissue culture were purchased from Greiner Bio-one, UK (6, 12, 24 and 96 well plates, 5, 10 and 25 ml pipettes, cell scrapers and cryovials) and PAA (15 and 50 ml Falcon tubes, 10 cm dishes, T25 and T75 flasks). 0.2 µm and 0.45 µm filters were purchased from Fisher Scientific, UK. Solutions and buffers were stored at room temperature unless specified. Cell culture media were stored at 4ºC.

2.1.1 Chemicals and solutions

All chemicals were purchased from Sigma-Aldrich, UK unless stated otherwise.

Ammonium persulfate (APS)

10%APS solution was made in dH_2O and stored at 4ºC for a maximum of two weeks.

Blasticidin S

Blasticidin 10mg/ml purchased from InvivoGen (cat# ant-bl-1)

Blocking buffer for western blotting

5 or 10% (w/v) Marvel milk powder (purchased from Sainsbury supermarket) in Tris-Buffered Saline-Tween20 (TBST)

Caspase inhibitor

20 mM caspase inhibitor Z-VAD-FMK, purchased from Promega (cat# G7231)

Coomassie Brilliant Blue staining solution for Western blotting

- 0.25% Coomassie Brilliant Blue G-250 (cat# 6104-58-1)

- 45% methanol
- 44.75% dH_2O
- 10% acetic acid

Coomassie Brilliant Blue destain

- 45% methanol
- 45% dH_2O
- 10% acetic acid

Coumaric acid

- 90 mM p-coumaric acid in Dimethyl Sulfoxide (DMSO)
- Stored at -20 ºC

Dulbecco's Modified Eagle Media (DMEM)

Purchased from PAA (Cat # E15-810) and supplemented before use with

- 10% fetal calf serum (FCS), PAA (cat# A15-151)
- 50 µg/ml streptomycin
- 100 µg/ml penicillin
- 1 mM sodium pyruvate

Dulbecco's Modified Eagle Media-Ham's F12 (DMEM-F12)

Purchased from Gibco BRL (cat# 21331-020) and supplemented before use with

- 4 mM L-Glutamine
- 25 µg/ml hydrocortisone
- 5% Fetal calf serum (FCS)
- 50 µg/ml streptomycin
- 100 µg/ml penicillin
- 1 mM sodium pyruvate

EDTA buffer (10x), pH 8.0

1 mM EDTA, made in dH_2O, pH was adjusted to 8.0 and the solution was stored at 4ºC

Enhanced Chemiluminescece (ECL) buffer

- 100 mM Tris-HCL (tris [hydroxymethyl] aminomethane hydrochloride) pH 8.5
- Stored at 4ºC

ELISA assay buffer

- 1x phosphate buffered saline (PBS)
- 0.5% Bovine Serum Albumin (BSA)
- 0.1% Tween 20
- Stored at 4ºC

ELISA kit for TNF-α

- Human TNF-α CytoSet™, 10 plates format purchased from Invitrogen (cat# CHC1753) and consisted of
- Coating antibody: anti-human TNF-α
- Detection antibody: anti-human TNF-α Biotin
- Standard: recombinant human TNF-α
- Streptavidin-HRP

ELISA wash buffer

- 1x phosphate buffered saline (PBS)
- 0.05% Tween 20

FACS buffer for flow cytometry

- 1x phosphate buffered saline (PBS)
- 4% fetal calf serum (FCS)

Freezing medium for cell culture

- 10% Dimethyl Sulfoxide (DMSO)
- 20% fetal calf serum (FCS)
- 70% Dulbecco's Modified Eagle Media (DMEM)
- Stored at 4ºC

G418 sulphate

Purchased from InvivoGen (Cat# ant-gn-1)

IZ-TRAIL

- Recombinant human isoleucine zipper trimerization TRAIL (Ganten et al., 2006)
- From Prof. Henning Walczak, Imperial College London

Keratinocyte Growth Medium-2 (KGM-2) BulletKit

- Purchased from Lonza (Cat# CC3107) and consisted of
- Keratinocyte basal medium, supplemented before use with;
- 0.5 ml Hydrocortisone
- 0.5 ml Transferrin
- 0.25 ml Epinephrine
- 0.5 ml Gentamicin, amphotericin (GA-1000)
- 0.5 ml Bovine pituitary extract (BPE)
- 0.5 ml Recombinant human epidermal growth factor (rhEGF)
- 0.5 ml Bovine insulin

Laemmli Sample Buffer

- 62 mM Tris base pH 6.8
- 10% glycerol
- 2% SDS
- 5% β-mercaptoethanol

Luria-Bertani (LB)-agar plates

- 1.5% (w/v) agar in Luria-Bertani medium
- Sterilised by autoclaving
- Cooled to 50ºC
- Appropriate antibiotic was added
- Poured in 10 cm plates

Luminol

- 250 mM luminol in DMSO
- Stored at -20ºC in the dark.

Luria-Bertani medium

- 1% NaCl
- 0.5% Bacto-yeast extract
- 1% Bacto-tryptone
- Made up in dH_2O
- pH was adjusted to 7.0 with 5M NaOH.
- Sterilised by autoclaving.

Midiprep solutions

Plasmid Midi Kit was purchased from QIAGEN (cat#12143)

Miniprep solution

Wizard® Plus SV Minipreps DNA Purification System was purchased from Promega (Cat# A1330)

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) stock

- 5 mg/ml MTT (Merck chemicals, cat# 475989)
- Prepared in phosphate buffered saline (PBS)
- Sterilised by filtering through a 0.2 µm filter

- Stored in -20ºC in dark

Opti-MEM reduced serum medium

Purchased from Invitrogen (cat# 31985047)

Phosphate –Buffered Saline (PBS)

- 140 mM NaCl
- 2.7 mM KCl
- 8.0 mM Na_2HPO_4
- 1.5 mM KH_2PO_4
- Made in dH_2O and sterilised by autoclaving.

Penicillin/Streptomycin (500x) stock solution

- 1.25 g Streptomycin sulphate
- 2.5 g Penicillin
- Made in 50 ml dH_2O and sterilised by filtering through a 0.2 µm filter

Phenylmethyl-sulfonyl fluoride (PMSF)

10 mM PMSF was made in isopropanol and stored at -20ºC.

Running/Transfer buffer (10x) for western blotting

- 250 mM Tris base
- 2.5 M Glycine
- 1% sodium dodecyl sulphate (SDS)
- Made in dH_2O

Running buffer (1x) for western blotting

- 10% 10x Running/Transfer buffer
- 90% dH_2O

SDS-Polyacrylamide Resolving gel 10% (10 ml)

- The reagents were added in order as follow

- 4 ml dH_2O
- 3.3 ml acrylamide mix (purchased from Fisher Scientific, cat# EC-890-1)
- 2.5 ml 1.5M Tris pH 8.8
- 1 ml 10% sodium dodecyl sulphate (SDS)
- 1 ml 10% ammonium persulphate (APS)
- 0.004 ml TEMED

SDS-Polyacrylamide Resolving gel 12% (10 ml)

- 3.3 ml dH_2O
- 4 ml acrylamide mix
- 2.5 ml 1.5M Tris pH 8.8
- 1 ml 10% sodium dodecyl sulphate (SDS)
- 1 ml 10% ammonium persulphate (APS)
- 0.004 ml TEMED

SDS-Polyacrylamide Stacking gel 5% (4 ml)

- 2.7 ml dH_2O
- 0.67 ml acrylamide mix
- 0.5 ml 1 M Tris pH 6.8
- 0.04 ml 10% sodium dodecyl sulphate (SDS)
- 0.04 ml 10% ammonium persulphate (APS)
- 0.004 ml TEMED

siRNA (small inhibitory RNA)

XIAP siRNA oligonucleotide was purchased from MWG Biotechnology

5’ AUCCAUCCAUGGCAGAUUA 3’

Smac mimetic (Smac59, SM-164)

- Bivalent Smac mimetic (Sun et al., 2007)
- From Prof Dominico Delia, Fondazione IRCCS Istituto Nazionale Tumori, Milan, Italy

Sodium citrate buffer pH 6.0

- 10 mM anhydrous citric acid
- 1M NaOH
- Made in dH_2O, pH was adjusted to 6.0 and the solution was stored at 4ºC

Sodium dodecyl sulphate (SDS) 10%

- 10% (w/v) solution of SDS was made in dH_2O
- pH was adjusted to 7.2 with concentrated HCl

Solubilisation solution for MTT assay

- 50% dimethylformamide
- 0.2% glacial acetic acid
- 20 mM HCl
- 10% SDS
- Made in dH_2O

Stripping buffer for western blotting

Re-Blot Plus (strong/mild) solutions were purchased from Millipore (cat# 2504/2502)

Tris-Buffered Saline TBS (10x)

- 250 mM Tris base
- 1.5 M NaCl
- Made in dH_2O
- pH adjusted to 7.4 with concentrated HCl
- sterilized by autoclaving

Tris-Buffered Saline-Tween 20 (TBST)

0.05% Tween 20 in 1x TBS

Tris/EDTA buffer pH 9.0

- 10 mM Tris Base
- 1 mM EDTA
- Made in dH2O
- pH was adjusted to 9.0
- Stored at 4ºC

Tris-EDTA (TE)

- 10 mM Tris-HCl pH 8.0
- 1 mM EDTA pH 8.0
- Made in dH_2O

TMB Substrate Kit

Purchased from Thermo Scientific (cat# 34021)

TNF-α (recombinant human)

- Recombinant human TNF-α
- Gift from Prof. Henning Walczak, Imperial College London.

Transfection Reagents

- X-tremeGENE HP DNA Transfection Reagent, purchased from Roche (cat# 06 366 244 001)
- X-tremeGENE siRNA Transfection Reagent, purchased from Roche (cat# 04 476 093 001)

Transfer buffer (1x) for western blotting

- 10% 10x running/transfer buffer
- 20% methanol
- 70% dH_2O

Trypsin (1x)

- 10% 10xTrypsin purchased from Sigma (cat# T4549)

- 90% versene

Versene

- 0.270 mM EDTA pH 8.0
- Made in PBS and sterilised by autoclaving

2.1.2 Antibodies

Primary antibodies listed by antigen

β-Actin

- Mouse monoclonal anti-β-Actin (AC-15)
- Provider: Sigma Aldrich (cat# A5441)
- Dilution: 1:5000 (Western Blotting)

Bcl-2

- Mouse monoclonal anti- Bcl-2 (100)
- Provider: Santa Cruz Biotechnology (cat# sc-509)
- Dilution:1:1000 (Western Blotting)

Caspase-3

- Rabbit polyclonal anti-caspase-3
- Provider: Cell Signaling (cat# 9662)
- Dilution: 1:1000 (Western Blotting)

Caspase-8

- Mouse monoclonal anti-caspase-8 (1C12)
- Provider: Cell Signaling (cat# 9746)
- Dilution: 1:1000 (Western Blotting)

CD 120a (TNF-R1)

- Mouse monoclonal anti-human CD120a (TNF-R1), clone H398
- Provider: ABD Serotec (cat# MCA2789Z)
- Dilution: 10 µg/ml, functional assays

cIAP-1

- Affinity-purified goat anti-human cIAP-1 (HIAP) Antibody
- Provider : RandD system (cat# AF8181)

- Dilution: 0.5 µg/ml (Western Blotting)

cIAP-2

- Purified mouse anti-human c-IAP-2
- Provider : BD Pharmingen (cat# 552782)
- Dilution: 4 µg/ml (Western Blotting)

EGFR

- Monoclonal mouse anti-EGFR (F4)
- Provider: Gift from Prof. William Gullick, Department of Bioscience, University of Kent at Canterbury, UK
- Dilution: 1:1000 (Western Blotting)

ERCC1

- Mouse monoclonal anti-ERCC1
- Provider: ABR-Affinity (cat# MA1-21734)
- Dilution: 1:50 (Immunohistochemistry)

c-FlIP

- Mouse monoclonal anti-human FLIP (NF6)
- Provider: Enzo Life Science (cat# ALX-804-428)
- Dilution: 1:500 (Western Blotting)

HIF-1α

- Mouse monoclonal anti-human HIF-1α
- Provider: Becton Dickinson (cat# 610958)
- Dilution: 1:50 (Immunohistochemistry)

HS101 and HS201

- Mouse monoclonal anti-TRAIL receptor 1 and 2 antibodies
- Provider: gift from Prof. Henning Walczak, Imperial college London
- Dilution: 5 µg/ml (flow cytometric analysis)

hTNFα-IgA

- Neutralizing IgA monoclonal antibody to human tumour necrosis factor alpha
- Provider: InvivoGen (ca# maba-htnfa)

Ik-Bα

- Mouse monoclonal anti-Ik-Bα (L35A5)
- Provider: Cell Signaling (cat# 4814)
- Dilution: 1:1000 (Western Blotting)

KU80

- Rabbit polyclonal anti-KU80
- Provider: Cell Signaling (cat# 2753)
- Dilution: 1:200 (Immunohistochemistry)

Mcl-1

- Rabbit polyclonal anti-Mcl-1
- Provider: Cell Signaling (cat# 4572)
- Dilution: 1:1000 (Western Blotting)

PARP

- Mouse monoclonal anti-cleaved PARP
- Provider: Cell Signaling (cat# 9548)
- Dilution: 1:1000 (Western Blotting)

PTEN

- Mouse monoclonal anti-human PTEN
- Provider: Dako (cat# M3627)
- Dilution: 1:50 (Immunohistochemistry)

P16

- Mouse ant-human p16 ^{INK4a} antibody

- CINTec Histology V-kit, cat# 9512

Rad51

- Mouse monoclonal anti-Rad51
- Provider: Abcam (cat# ab54188)
- Dilution: undiluted (Immunohistochemistry)

Smac/DIABLO

- Rabbit polyclonal anti-human-SMAC/DIABLO
- Provider: Epitomics (Cat# 1012-1)
- Dilution: 1:1000 (Western Blotting)

XIAP

- Purified mouse monoclonal anti-XIAP
- Provider: BD Transduction Laboratories (cat# 610762)
- Dilution: 1:4000 (Western Blotting)

XRCC1

- Mouse monoclonal anti-XRCC1
- Provider: Abcam (cat# ab1838)
- Dilution: 1:50 (Immunohistochemistry)

Secondary antibodies listed by source species

Anti-mouse peroxidase based Dako EnVision system- HRP-DAB

- Provider: Dako (cat# K4007)
- Used for Immunohistochemistry with mouse monoclonal primary antibodies

Anti-Rabbit peroxidase based Dako EnVision system- HRP-DAB

- Provider: Dako (cat# K4011)
- Used for Immunohistochemistry with rabbit polyclonal primary antibodies

Donkey-anti-goat IgG secondary antibody conjugated to horseradish peroxidase

- Provider: RandD System (cat# HAF109)
- Dilution: 1:1000 (Western Blotting)

Donkey-anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase

- Provider: Fisher scientific (cat# NA934)
- Dilution: 1:2000 (Western Blotting)

Goat-anti-mouse IgG secondary antibody conjugated to horseradish peroxidase

- Provider: Sigma Aldrich (cat# A0168)
- Dilution: 1:4000 (Western Blotting)

2.1.3 Cell lines

HSC3

- Human head and neck squamous cell carcinoma (HNSCC) cell line
- Gift from Dr Kazuya Tominaga, Department of Oral Pathology, Osaka Dental University. Japan

HSC3M3

- HNSCC cell line
- Gift from Dr Kazuya Tominaga, Department of Oral Pathology, Osaka Dental University. Japan

HN5

- HNSCC cell line
- Gift from Dr Barry Gusterson, Department of Pathology, University of Glasgow, UK

HN30

- HNSCC cell line
- Gift from Dr Andrew Yeudall, Virginia Commonwealth University, school of Dentistry Philips Institute of Oral and Craniofacial Molecular Biology. USA

H357

- HNSCC cell line
- Gift from Dr Stephen Prime, Department od Oral and Dental Science, University of Bristol. UK

UMSCC74A

- HNSCC cell line
- Gift from Dr Thomas E. Carey, University of Michigan, Ann Arbor. USA

UMSCC74B

- HNSCC cell line
- Gift from Dr Thomas E. Carey, University of Michigan, Ann Arbor. USA

UMSCC11B

- HNSCC cell line

- Gift from Dr Thomas E. Carey, University of Michigan, Ann Arbor. USA

UMSCC22B

- HNSCC cell line
- Gift from Dr Thomas E. Carey, University of Michigan, Ann Arbor. USA

Normal Human Keratinocyte (NHK)

- Primary Oral keratinocyte
- Gift from Prof. Paul M. Speight, Department of Oral Pathology, School of Clinical Dentistry, University of Sheffield

HaCat

- Spontaneously immortalized Keratinocyte cell line
- Gift from Prof P Morgan, Department of Oral Pathology, Dental Institute, King's College London. UK

H357-bcl2

- H357 cells stably expressing Bcl-2
- Gift from Dr Marcella Flinterman. Head and Neck Oncology group, Dental Institute, King's College London. UK

HSC3M3- bcl2

- HSC3M3 cell line stably transfected with Bcl-2 expression vector
- Constructed for this research project

UMSCC11B-sh-XIAP and UMSCC-sh-LacZ

- UMSCC11B cell line stably transfected with Plenti6/V5 DEST-sh-XIAP or Plenti6/V5 DEST-sh-lacZ plasmid
- Constructed for this research project

2.1.4 Plasmids

Bcl-2-pcDNA3

- pcDNA3 plasmid expressing human bcl-2
- From Prof. Christoph Borner, Institute of Molecular Medicine and Cell Research, Albert-Ludwigs-University of Freiburg, Germany

PLenti6-sh-XIAP

- Plenti6/V5-DEST plasmid containing inhibitory sequence against h-XIAP
- From Prof. Hamid Kashkar, Institute for Medical Microbiology, Immunology and Hygiene, University of Cologne, Germany

PLenti6-sh-lacZ

- Plenti6/V5-DEST plasmid containing inhibitory sequence against Lac-Z
- From Prof Dominico Delia, Fondazione IRCCS Istituto Nazionale Tumori, Milan, Italy

2.2 Methods

2.2.1 Cell culture

Unless otherwise specified, all cells were maintained at standard culture conditions (37ºC and 5% CO_2)

2.2.1.1 Cell line maintenance

HaCat, HSC3, HSC3M3, HN5, HN30, UMSCC11B, UMSCC22B, UMSCC74A and UMCSCC74B were cultured in DMEM supplemented with 10% fetal calf serum (FCS), 50µg/ml streptomycin, 100µg/ml penicillin and 1mM sodium pyruvate and incubated at 37ºC and 5% CO_2. H357 was cultured in DMEM-F12 supplemented with 5% FCS, 4mM L-Glutamine, 25µg/ml hydrocortisone, 50µg/ml streptomycin, 100µg/ml penicillin, and 1mM sodium pyruvate. NHK were cultured using keratinocyte growth medium-2 bulletKit which consists of Keratinocyte Basal Medium (KBM) supplemented before use with Bovine Pitutary Extract (BPE), human Epidermal Growth Factor (hEGF), bovine insulin, Hydrocortizone, GA-1000 (Gentamicin, Amphtericin), Epinephrine and Transferrin (all these reagents were supplied with the kit). For Bcl-2 expressing cells, G418 was added at 400µg/ml for selection. For Plenti-sh-XIAP and Plenti-sh-LacZ cells, Blasticidin was added at 5µg/ml for HSC3M3 cells and at 10µg/ml for UMSCC11B cells.

2.2.1.2 Passaging of cell lines

Cells were sub-cultured every 3-5 days or when confluent. The old medium was removed and the cells were washed with 5ml versene and then incubated with 1x trypsin at 37ºC and 5% CO_2 for 1-5 min depending on cell line. Once cells became rounded and detached from the plates, they were re-suspended in fresh medium and seeded at the desired density.

2.2.1.3 Freezing and thawing of cell lines

Cells were trypsinized as mentioned in 2.2.1.1 and centrifuged for 5 min at 900g in IEC Centra-4x centrifuge, international equipment company, UK. The cell pellet was resuspended in 1ml freezing medium, transferred to a cryovial and stored in container filled with isopropanol to control the freezing speed and kept at -70ºC for 24 hours before being moved to liquid nitrogen. To thaw the cells, they were taken from liquid nitrogen, thawed in the vial in a 37ºC water bath and plated in 10 cm dishes with 9ml of fresh medium pre-warmed to 37ºC and incubated at 37ºC and 5% CO_2. Fresh medium was added 24 hours later.

2.2.1.4 Cell counting

The cells were trypsinized and resuspended in fresh medium. 10µl of cell suspension was pipetted into the counting chambers of a haemocytometer (SLS, cat# HAE2118) and the number of cells in the outer 4 large squares was counted. The concentration of cells/ml was the mean of cells number in the 4 squares multiplied by 10^4.

2.2.2 MTT cell viability assay

To assess the viability of cell population in response to different treatments, cells were seeded in 96 well plates at a density of 2-4 $x10^3$ cells in 100µl medium/well. After 24 hours, the medium was removed and the drug was dissolved in fresh medium and 100µl was added to cells. The cells were incubated for 24-72 hours at 37ºC and 5% CO_2. Then 20µl of MTT solution was added to each well, incubated with cells for 2-4 hours at 37ºC and 5% CO_2 before adding 150µl of solubilisation solution to each well. The plates were incubated for a further 16-24 hours and the optical density $(OD)_{595}$ was measured using LT-400 micro-plate reader, Labtech, using Manta software. The results were calculated after background subtraction by comparing the readings for treated cells in relation to control untreated cells.

2.2.3 Western blotting

2.2.3.1 Protein extraction

In order to lyse the cells for protein extraction, growth medium was removed, and the cells were washed twice with 5ml cold PBS. Cells were lysed by adding Laemmli sample buffer and scraping the cells with sterile disposable cell scraper (Greiner Bio-one). Lysates were kept on ice until use or at -20ºC for long term storage. Lysates were homogenized using Kontes Pellet Pestle Micro Grinders (Cat #SF24837V) and boiled for 5 min before loading onto SDS-PAGE gel.

2.2.3.2 SDS-polyacrylamide gel preparation and electrophoresis

The vertical gel-casting apparatus was set up according to the manufacturer's instructions (Bio-Rad, Mini PROTEIN II, cat# 165-2940). The resolving polyacrylamide gel solution was prepared and poured. 200µl of 100% ethanol was added on top of the gel to flatten its surface. After the gel had set the ethanol was removed with filter paper, then the gel was washed with dH_2O and dried with filter paper. The stacking gel was prepared and poured on top of the resolving gel and the comb was inserted. After the gel had set, the comb was removed and the gel was placed into the electrophoresis apparatus. The gel tank was filled with 1x running buffer and equal amounts of protein were loaded in each well.The first lane was loaded with 4µl of protein molecular weight marker (Fermentas, cat# SM0671). Electrophoresis was performed for 1.5-2 hours at 80V.

2.2.3.3 Coommassie Brilliant Blue staining

5µl of lysate of each sample was run in SDS-PAGE gel. Then the gel was incubated with Coommassie Brilliant Blue stain for at least 4 hours and destained with Coommassie Brilliant Blue destain for 24 hours on a rocking table. By comparing the intensity of the protein staining, equal amounts of protein could be loaded for protein expression testing.

2.2.3.4 Transfer of proteins onto a nitrocellulose membrane

Proteins were transferred onto 0.45µm pore nitrocellulose membrane (Schleicher and Schuell, cat# BA85-401191). In a tray filled with 1x transfer buffer, a soft scouring pad was put onto the black side of a transfer cassette, followed by 2 pieces of 3mm filter paper (Whatman, cat# 3030917), the gel, the nitrocellulose membrane and another 2 pieces of filter paper, all cut to the same size as the gel. A soft scouring pad was put on top, the transfer cassettes were closed and placed in the transfer tank (Bio-Rad Mini PROTEIN II) filled with 1x transfer buffer. Transfer was performed at 50V for 2-3 hours at 4ºC or 40mA overnight at room temperature.

2.2.3.5 Probing of western blot and detection

The antibodies used for western blot analysis were directed against β-Actin, Bcl-2, Caspase-3, Caspase-8, cIAP-1, c-IAP-2, EGFR, Ik-Bα, Mcl-1, FLIP, PARP, Smac/DIABLO and XIAP. The blots were blocked for 30min in 5% blocking buffer followed by incubation with primary antibody diluted in 5% blocking buffer at concentrations mentioned at 2.1.2 for 1.5 hour at room temperature or overnight at 4ºC on a rocking table . The blots were then rinsed 3times in TBST and washed once for 15min and 2times for 5min in TBST. Then the blots were incubated with secondary anti-mouse, anti-rabbit or anti-goat antibodies diluted in 5% blocking buffer. Finally the blots were rinsed three times in TBST, washed for 1 hour followed by further 2washes of 5min each in TBST.

2.2.3.6 Enhanced Chemiluminescence detection method (ECL)

10ml of the ECL buffer was first mixed with 3µl H_2O_2, then with 25µl coumaric acid and 50µl luminol. The blots were incubated with ECL solution for 1min, drained and wrapped in cling film and exposed to RX autoradiograph film (Fuji) from 5sec to 20min. Before re-probing with another primary antibody, the blot was stripped with stripping buffer for 15min and incubated with blocking buffer 2times for 5min each.

2.2.4 Enzyme Linked ImmunoSorbent Essay (ELISA)

ELISA test was used for detecting the secreted TNF-α in cells growth medium, to prepare samples for ELISA, an equal number of cells under investigation were seeded in 1ml medium in 12well plates, incubated at 37ºC and 5% CO_2. After 24 hours, medium was replaced with fresh medium to which TRAIL 50ng/ml or Smac59 50nM were added, and incubated for further 24 hours. The medium was removed and stored at -70ºC until it was used to measure the level of secreted TNF-α using ELISA.

ELISA was performed according to manufacture instructions, briefly, the capture/coating antibody (anti-human TNF-α) was diluted 1:1000 to a final concentration of 2µg/ml in PBS and 100µl was added to each well in a 96well polysterene plate (Nunc Immunosorp, cat# 475094). The plate was incubated overnight at 4ºC in a humid chamber, supernatant aspirated and the wells were washed once with ELISA wash buffer. 250µl of ELISA assay buffer was added to each well. The plate was then incubated at room temperature for 1-2hours. Assay buffer was aspirated and the wells were washed once with ELISA wash buffer. Test samples of cell medium and standards of recombinant human TNF-α, was added in 100µl to designated wells and were then incubated at room temperature for 2hours. Then samples and standard were aspirated, wells were washed 3times with ELISA wash buffer. 100µl of detection antibody (1:1250 in ELISA assay buffer) was added to each well and the plate was incubated at room temperature for further 2 hours. Wells were then washed 3times, and 100µl of streptavidin-HRP solution (diluted 1:1000 in ELISA assay buffer) was added to each well and incubated at room temperature for 30min. Wells were washed 3times, and 50µl TMB substrate was added and the plate was incubated in the dark for 10-20 min or until the colour developed. Finally 50µl of 2N H_2SO_4 stop solution was added and the colour intensity was measured at OD_{450} using an LT-4000 micro plate reader and Manta software.

2.2.5 Transformation of plasmid DNA into competent bacterial cells

2µl of plasmid DNA in Tris-EDTA buffer was added to 100µl of competent cells (One-Shot mach1 T1, Invitrogen, cat# C8620-03). The mixture was mixed by flicking the tube gently 2-3times. The cells were incubated on ice for 30min, transferred to a heat block at 42ºC for 30sec and back on ice for 2 min. 400µl of LB medium was added and the cells were then incubated in water bath at 37ºC for 1hour. The cells were spun at 13000g for 1min and 300µl of supernatant was discarded. Cells were resuspended in the remaining medium and 50µl of this suspension was plated on an agar plate containing the appropriate selection antibiotic (carbencillin or kanamycin) and were incubated overnight at 37ºC. A single colony was used to inoculate LB medium for mini- or midi-prep plasmid isolation.

2.2.6 Plasmid mini-prep

10ml of LB medium containing either 50µg/ml carbencillin or 25µg/ml kanamycin as appropriate was inoculated with a single bacterial colony, grown overnight at 37ºC with shaking at 220 rpm. The mini-prep was performed using the Wizard® Plus SV Minipreps DNA Purification System (Promega) following centrifugation protocol recommended by the manufacturer. Briefly, the overnight culture was centrifuged at 6000g for 5min to harvest the bacterial cells, and then the cleared lysate was produced by adding 250µl cell suspension solution, 250µl cell lysis solution, 10µl alkaline protease and 350µl neutralization solution to the bacterial cell pellet and centrifuged at 13000g for 10min at room temperature. Binding of plasmid DNA to column was performed by centrifugation of the cleared lysate within the column at 13000g for 1min at room temperature. Then the column was washed by adding 750µl wash solution and centrifuged at 13000g for 2min at room temperature. Finally the plasmid DNA was eluted by 100µl of nuclease free water to the spin column and centrifuged at 13000g for 1 min at room temperature then stored at -20ºC until the DNA concentration was measured using NanoDrop spectrophotometer (Thermo Scientific).

2.2.7 Plasmid midi-prep

5ml of LB medium containing either 25µg/ml carbencillin or 12.5µg/ml kanamycin as appropriate was inoculated with a single bacterial colony, grown for 6-8 hours at 37ºC with shaking at 220rpm. Then 100µl of this culture was added to inoculate 50ml LB medium containing appropriate antibiotic and grown overnight at 37ºC with shaking at 220rpm. Large quantities of plasmid DNA were purified using DNA purification columns from a midi-prep kit (QIAGEN) as recommended by the manufacturer. Briefly, the bacterial cells were harvested by centrifuging 50ml of overnight culture at 6000g for 15min at 4ºC, then the bacterial pellet was resuspended in 4ml resuspension buffer, then 4ml lysis buffer was added and the cells were incubated for 5min at room temperature. 4ml of neutralization buffer was added to the previous mixture and incubated for 15min on ice before it was centrifuged for 1hour at 2000g and 4ºC. 4ml equilibration buffer was passed through the purification column before applying the supernatant to pass through the column. The plasmid DNA was then eluted using 5ml elution buffer, then DNA was precipitated by adding 3.5ml isopropanol and centrifuged at 5000g for 1hour at 4ºC. Supernatant was then removed and pellet was washed with 70% ethanol and centrifuged at 5000g for 1hour at 4ºC. Supernatant was removed and pellet was air dried and then dissolved in 150µl TE buffer and stored at 4ºC until measuring DNA concentration using NanoDrop.

2.2.8 Transfection using X-tremeGENE HP DNA Transfection Reagent

The transfection was performed using X-tremeGENE HP DNA Transfection Reagent blend of lipids that complexes with and transports DNA into cells as recommended by the manufacturer (Roche). Cells were plated 24hours before transfection in 12well plates in growth medium and incubated at 37ºC and 5% CO_2. 1.0µg of plasmid DNA was added to 100µl Opti-MEM reduced serum medium and mixed gently, and then 2µl of X-tremeGENE HP DNA Transfection Reagent was added to the diluted DNA mixture and mixed gently. The mixture was incubated at room temperature for 15-30min to allow X-tremeGENE HP Reagent:DNA complex

formation. The mixture was added drop-wise to different areas of the well. The plate was gently rocked back-and-forth and side-to-side to evenly distribute the X-tremeGENE HP Reagent:DNA complex. The cells were then incubated at 37ºC and 5% CO_2 for 24-72hours until tested.

2.2.9 Transfection using X-tremeGENE siRNA Transfection Reagent

siRNA transfection was performed using X-tremeGENE siRNA Transfection Reagent (Roche). Cells were plated 24 hours before transfection in 24 well plate in growth medium and incubated at 37ºC and 5% CO_2. The transfection reagent: siRNA complex was prepared in separate eppendorf test tubes; the first was to dilute 10µl of the X-tremeGENE siRNA transfection reagent in 40µl of Opti-MEM serum reduced medium. The second was to dilute 2µg of siRNA in Opti-MEM serum reduced medium to a total volume of 50 µl. The content of both tubes was mixed within 5min, and incubated for 15-20min at 15-25ºC to allow X-tremeGENE HP Reagent:siRNA complex formation. The mixture was then added drop-wise to the cells and the plate was swirled to ensure complete and even distribution. The cells were incubated with the mixture for 24 to 48 hours and the gene knockdown was tested using western blotting.

2.2.10 Cloning of cells

2.2.10.1 Titration of antibiotic stocks (kill Curves)

Prior to using G418 and Blasticidin to establish stable cell lines, the optimal concentration of the selectable marker for each cell line was determined. Cells were plated in 12well plates containing 1ml of complete growth medium with either 0, 50, 100, 200, 400 and 800µg/ml of G418 or 0, 5, 7 and 10µg/ml of Blasticidin. The cells were incubated at 37ºC and 5% CO_2 for 10-14 days, the selective medium was replaced every 4days and the cells were checked for cell death every 2days. The concentration used for selection was the lowest concentration that gave detectable cell death in up to 5days and killed all the cells within two weeks.

2.2.10.2 Establishment of stably transfected cell lines

Cells were plated and transfected with the construct of interest as mentioned in 2.2.5. After 24 hours, the medium was replaced with fresh medium supplemented with the selection antibiotic G418 or Blasticidin at a concentration previously determined to be optimal (section 2.2.10.1). The cells were incubated at 37ºC and 5% CO_2 for 1-2weeks during which medium was changed every 2days. Most of the cells were killed and washed off the bottom of the plate during medium changing, leaving colonies of stably transfected cells. The stable colonies were collected by trypsinization and transferred to T25 flasks for expansion. The cells were tested for successful transfection using western blotting.

2.2.11 Flow cytometric analysis (FACS) for TRAIL receptor expression

In order to test TRAIL receptor expression on HNSCC cell membrane using flow cytometry, cells to be tested were washed twice with PBS, trypsinized and resuspended in fresh medium to be counted as mentioned in section 2.2.1.4. 400,000 cells in 400µl fresh medium were added to each well in 96well tissue culture plate. The cells were pelleted by centrifugation at 3000rpm for 3 min. The supernatant was discarded and the pellet was resuspended in the primary antibody (HS101 anti-TR1 or HS201 anti-TR2) diluted to 5µg/ml in FACS buffer and incubated on ice for 30 min. The cells were then spun at 3000rpm for 3 min, and washed 3 times with ice cold FACS buffer by adding 100µl of buffer and centrifuged at 3000rpm for 3min each time. The secondary antibody diluted to 1:200 in FACS buffer was added to the cells and incubated for 20min on ice. As above, cells were spun for 3min and washed 3times with ice cold FACS buffer. The Streptavidin-PE coupled antibody diluted 1:200 in FACS buffer was added to the cells and incubated on ice for 20min in the dark. As above, cells were spun for 3min and washed 3 times with ice cold FACS buffer. The cells were then resuspended in 100µl of PI solution (1µg/ml, diluted in FACS buffer). Number of cell surface binding to the tested antibodies was analyzed by FACS. This experiment was performed in Prof. Henning Walczak laboratory, Imperial College London as a part of the collaboration.

2.2.12 Gene and mi-RNA expression profiling

HNSCC cells were seeded in 10cm dishes and 24 and 48 hours later, the cells were trypsinized and resuspened in fresh medium and were centrifuged at 900g for 5 min. The cell pellets then were mixed with 1ml Trizol (Invitrogen, cat# 15596026) and were stored at -80ºC. Samples were then shipped to Fondazione IRCCS Istituto Nazionale Tumori, Milan, Italy for performing RNA extraction and expression profiling analysis. Gene expression profiling was performed using the Illumina Human HT-12 v3 Expression BeadChip, which targets more than 25,000 annotated genes with more than 48,000 probes. miRNA expression profiling was performed using the Illumina Human v2 miRNA Beadchip which detects 1,146 of known human miRNAs.

Data obtained were imported into BeadStudio and were processed and analyzed by Katherine Lawler, bioinformatics analyst at KCL/UCL Comprehensive Cancer Imaging Centre, King's College London. Raw probe-level intensity values, detection *p*-values and bead counts were exported from GenomeStudio V2010:1 (miRNA) / BeadStudio V3 (mRNA) and annotated using Illumina manifests HumanMI_V2_R0_XS0000124-MAP.bgx / HumanHT-12_V3_0_R1_11283641. bgx. Arrays were quantile normalized using the R/Bioconductor package beadarray (v2.0.2). The hierarchical clustering (dendrograms) indicated on expression heatmaps were obtained using complete linkage agglomerative clustering based on Euclidean distance Where multiple probes on the array mapped to the same gene symbol, only the most variable probe is displayed (highest standard deviation). Differential expression between sample groups was called using limma (Smyth, 2004) empirial Bayes moderated *t*-tests *(because of small numbers of samples in the sample groups used for comparison, especially HN5 vs. HN30)*, at a false discovery rate (FDR)-corrected *p*-value threshold of 0.05.

2.2.13 Patient selection

Tissue biopsies from 150 head and neck squamous cell carcinoma (HNSCC) cases were selected from the pathology archives of the Oral Pathology Department,

Dental Institute, King's College London from patients treated in the period 1997 to 2004. The ethics approval is covered by existing project approval (REC reference, 04/02/10). The cases were selected to be representative of a range of primary sites. Original diagnostic slides were reviewed and blocks selected if they had enough tumour tissue to be used for construction of tissue microarray (TMA) blocks. The cases were categorized according to the site of the primary tumour. 27 cases were from the lateral border of the tongue, 17 from the floor of the mouth, 18 from the alveolus, 17 from the buccal mucosa, 17 from the lip vermillion border, 19 from the tonsils, 16 from the pharynx and 19 were from palate, tuberosity and retromolar area. Clinical and histological data for these cases were obtained from pathology reports and laboratory records of processing, follow up data bases maintained by the Department of Oral and Maxillofacial surgery and Radiotherapy departments and the central patient index of Guy's and St Thomas NHS foundation trust. Age at diagnosis, sex, site of occurrence, stage of the tumour, histological grade, presence of metastasis, time and type of treatment (surgery alone, surgery and radiotherapy or radiotherapy alone), time of recurrence and time of death were recorded on Excel spreadsheets, anonymised and stored in encrypted format according to NHS information governance requirements.

2.2.14 Tissue Microarray construction (TMA)

Haematoxylin and eosin (H&E) slides used for diagnosis of each case were reviewed to select representative carcinoma and dysplasia tissue areas to be used to construct the tissue microarray blocks. From each case a minimum of four and a maximum of eight area cores were selected according to tissue availability. The selected areas included four samples of carcinoma, and if available up to four additional cores of normal epithelium, epithelial dysplasia and up to two of metastatic carcinoma from two different lymph nodes. The selected areas were marked on the H&E slides and overlaid the respective paraffin blocks to identify the area to be sampled. The TMA recipient block was designed to include eight cases; the cores from each case were aligned vertically in one column. In addition, four cores of irrelevant animal tissues were placed on top left side of the array for

orientation (Figure 2-1A). Donor blocks were re-embedded to standard mould depth if the original blocks were of different thicknesses.

TMA blocks were constructed on the semi-automated Alphelys MTA Booster machine at the Breast Cancer Unit, Guy's Hospital using 1.0mm manual tissue arrayer punches (Beecher Instrument, Inc. cat# MP_{10}) (Figure 2-1B). Tissue cores four mm long were punched from the donor blocks and inserted in four mm deep holes created by the recipient punch in the recipient block. After all cores were inserted, the blocks were incubated at 56ºC in an oven for 15min in order to enhance the adhesion between the tissue cores and the paraffin of the recipient blocks.

18 TMA blocks were constructed, three for carcinoma of the lateral border of tongue, one for the lips, two for the floor of the mouth, two for alveolus, two for the buccal mucosa, two for the tonsils, two for the pharynx and two for palate, tuberosity and retromolar area. Additional two blocks were required for extra cases that could not be included into the original blocks due to cases number limitation.

2.2.15 Immunohistochemistry (IHC)

The TMA blocks were sectioned at 4µm sections using routine histological procedures on a LEICA-RM2235 microtome. Sections were cut by BMS staff at the KCL Department of Oral Pathology within three days of the staining and stored until use. For each TMA, two sections were used for immunohistochemical staining using five primary antibodies directed against; ERCC1, HIF-1α, KU80, PTEN, Rad51 and XRCC1 using methods recommended by manufactures when available. All were optimized for tissues under investigation.

Paraffin sections were deparaffinised by immersion in xylene with agitation for five min (twice) followed by immersion in absolute alcohol for five min (twice). Then the sections were washed with dH_2O for two min. To block the endogenous peroxidase the sections were immersed in 3% H_2O_2 for 15min, and then washed with dH_2O for five min. The antigen retrieval was performed by immersing the sections in the appropriate buffer in the microwave at 800W for 20min. The buffer

differs according to the antibody used; sodium citrate buffer pH 6.0 was used for ERCC1, KU80, PTEN and Rad51 antibodies. Tris/EDTA buffer pH 9.0 was used for XRCC1 antibody. 1mM EDTA buffer pH 8.0 was used for HIF-1α antibody. The sections were left to cool down, transferred to humified chamber and the primary antibodies were diluted in phosphate buffered saline and added on top of the tissue sections, and incubated either at room temperature or 4ºC for the required incubation period for each antibody as mentioned in section 2.1.2. The sections then washed in PBS for 15 min. Then Dako EnVision kit (anti-mouse or anti-rabbit as appropriate) were used to visualize the antibody staining. Finally, the sections were counterstained with haematoxylin and mounted in Distyrene Plasticizer Xylene (DPX). For each antibody a positive and a negative control section were used as follows; ERCC1 antibody was used at 1:50 dilution and incubated for overnight at 4ºC and thyroid carcinoma tissue was used as a positive control. HIF1-α antibody was used at 1:50 dilution and incubated for overnight at 4ºC and thyroid carcinoma tissue was used as a positive control. KU80 antibody was used at 1:200 dilution and incubated for overnight at 4ºC and oral squamous cell carcinoma tissue was used as a positive control. PTEN antibody was used at 1:50 dilution and incubated for overnight at 4ºC and breast cancer tissue was used as a positive control. Rad51 antibody was used undiluted and incubated for 3hours at room temperature and oral squamous cell carcinoma tissue was used as a positive control. XRCC1 antibody was used at 1:50 dilution and incubated for one hour at room temperature and oral squamous cell carcinoma tissue was used as a positive control.

2.2.16 Scoring of the immunohistochemistry staining

The expression of each antibody was scored by consensus between two pathologists blind to clinical and pathological data. Two parameters were scored for each antibody, the intensity of the staining (1=no stain, 2=equivocal, 3=weak, 4=moderate or 5=strong) and the percentage of the stained cells (1=0-25%, 2=25-50%, 3=50-75% or 4=75-100%). Four carcinoma cores were scored for each case and a final combined score was calculated as the average of the eight scores. The scores were recorded in the spreadsheet with the clinical and the histological data

for each case and imported into the IBM SPSS statistics 20 programme for statistical analysis.

Because of low number of cases for each category in the scoring system, the multiple scoring system of antigen expression has been transformed into binary scoring system before the analysis. Staining intensity scores were transformed into either negative (which included 1=no stain, 2=equivocal) or positive (which included 3=weak, 4=moderate or 5=strong). Percentage of the stained cells was transformed to either low (which included 1=0-25%) or high (which included 2=25-50%, 3=50-75% or 4=75-100%) expression.

Descriptive analyses were used to summarize clinical and histopathological characteristics of the study group. Cox regression was used to identify the significant predictors of overall survival and disease free survival. The association between antigen expression and response to treatment was tested using Chi-square/exact test. Logestic regression analysis was carried out to identify the significant predictors of response. The analysis was performed under the supervision of Dr Ana Nora Donaldson BSc MSc PhD CStat, statistical analyst for Kings' College London Dental Institute.

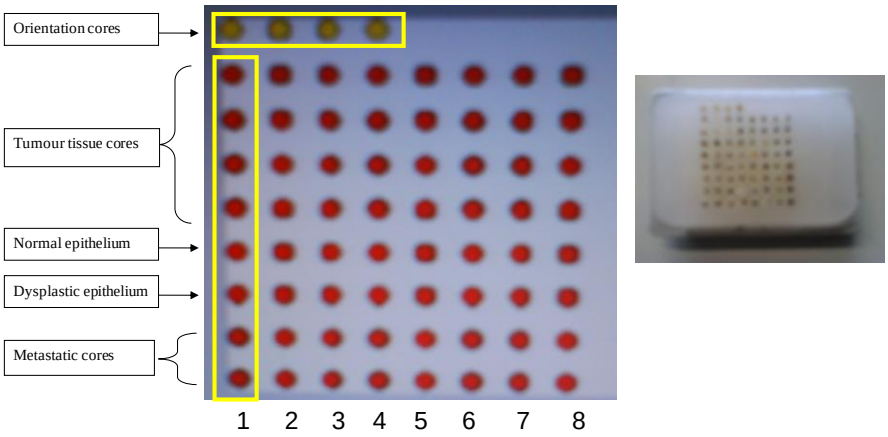
2.2.17 P16 immunostaining and HPV in situ hybridization (ISH)

TMA sections of tonsil and pharyngeal carcinomas were immunostained for detection of p16 protein. P16 IHC was carried out using a proprietary kit (CINtec Histology, mtm Laboratories) on a Ventana Benchmark Autostainer (Ventana Medical Systems). A tonsil SCC with high P16 expression was used as a positive control and same section without adding the p16 primary antibody as a negative control.

Eight cases of tonsil carcinomas were P16 positive, and whole FFPE sections of these cases were used for performing HPV DNA ISH. High-risk HPV DNA ISH was carried out using proprietary reagents (Inform HPV III Family 16 Probe (B), Ventana Medical Systems) on a Benchmark Autostainer (Ventana

Medical Systems). The Inform HPV III Family 16 Probe (B) detects high-risk genotypes HPV-16, -18, -31, -33, -35, -39, -51, -52, -56, -58 and -66.

A



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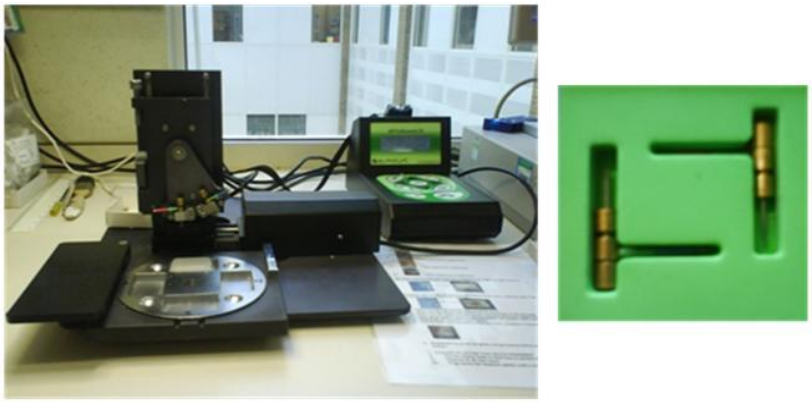


Figure 2.1 Design and equipment used for tissue microarray construction

A-left; the template design for each TMA included eight cases, each in a single column. In each column, the eight cores from each case were four cores of carcinoma, one core of normal epithelial tissue, one core of dysplastic epithelial tissue and two cores of metastatic tissue from regional lymph nodes. Four orientation cores made from animal tissue were included in the top left of the TMA block. A-right; A TMA block. B-left. Alphelys MTA Booster equipment. B- right 1.0 mm manual tissue arrayer punches, Beecher Instrument.

3 Results I

3.1 Effect of TRAIL and Smac mimetics on the viability of HNSCC cell lines

TRAIL and Smac mimetics are important candidates as anticancer agents because of their specific activity against cancer cells. TRAIL has been shown to effectively induce apoptosis in a range of tumour cell lines including leukaemia, multiple myeloma, neuroblastoma, lung, liver, colon, breast, prostate, pancreas, kidney and thyroid cancer in vitro (Newsom-Davis et al., 2009) as well as mouse models in vivo (Walczak et al., 1999). Recombinant TRAIL (AMG951, Apo2L/TRAIL) has proved to be effective when used in Phase I trials on patients with advanced solid and haematological malignancies (Herbst et al., 2010). Several reports have demonstrated that Smac mimetics are effective at inducing cell death in vitro and in vivo, both as a single agent and/or synergistically with other pro-apoptotic stimuli (Petersen et al., 2010). The effect of these two promising drugs on the HNSCC remains unknown. In this study, the response of HNSCC cell lines to TRAIL and Smac mimetics was investigated to determine whether these drugs could be potential candidates for treating HNSCC.

3.1.1 TRAIL and Smac59 titration in HNSCC cell lines

To test the cytotoxic effect of recombinant IZ-TRAIL and Smac59 on the viability of HNSCC lines, a panel of 9 cell lines (HSC3, HSC3M3, HN5, HN30, UMSCC74A, UMSCC74B, UMSCC11B, UMSCC22B and H357) were used. These cells represented HNSCC from different sites and different molecular features. The details of the molecular characteristics of these cell lines are shown in Table 3.1. The cells were seeded in 96 well plates at a density of 500-2000 cells/well. After 24 hours incubation at 37ºC and 5% CO_2 the cells were treated with different concentrations of TRAIL (100, 50, 25, 12.5, 6.25, 3.125 and 1.65 ng/ml) and Smac59 (200, 100, 50, 25, 12.5, 6.25 and 3.125 nM) for 72 hours. The cell viability was measured using MTT assay. IC_{50} (the concentration of the tested drug

that induced 50% cell death) was calculated for each cell line using GraphPad Prism5.

HSC3, HSC3M3 and HN5 cell lines were highly sensitive to Smac59 showing similar levels of cell death at both the lowest concentration (3.125 nM) and the highest concentration (200 nM) tested which made the IC_{50} calculation for these cell lines not possible at this dose range (data not shown). This suggested that a lower concentration range for Smac59 needed to be used for these cell lines (2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125 nM).

The results showed that HSC3, HSC3M3 and HN5 cells were sensitive to Smac59 and resistant to TRAIL. HN5 showed only 20% loss of viability when treated with 100 ng/ml TRAIL (Figure 3.1). The degree of sensitivity to Smac59 was different among the three cell lines with HSC3 being the most sensitive. The relative degree of sensitivity was reflected by the IC_{50} values which were 0.03639, 0.4712 and 0.2531 for HSC3, HSC3M3 and HN5 respectively. With the partial exception mentioned above, the three cell lines were resistant to TRAIL even at the highest concentration tested.

The results in Figure 3.2 showed the dose response of HN30, UMSCC74A and UMSCC74B for Smac59 and TRAIL. The results indicate that these three cell lines were resistant to Smac59 at all the concentrations tested with the exception of HN30 cells which showed only 20% loss of cell viability when treated with Smac59. HN30 was not considered to be Smac59 sensitive cells because its noted sensitivity to Smac59 was very low in comparison with the sensitivity of HSC3, HSC3M3 and HN5 cells. Conversely, these three cell lines showed marked sensitivity to TRAIL. HN30 cell line was very sensitive with an IC_{50} value of 6.759 ng/ml. UMSCC74B was more sensitive than UMSCC74A with IC_{50} of 28.31 and 82.1ng/ml respectively.

The sensitivity or resistance of H357, UMSCC11B and UMSCC22B to TRAIL and Smac59 was similar to HN30, UMSCC74A and UMSCC74B (Figure 3.3), data showing resistant to Smac59 and sensitivity to TRAIL. The degree of sensitivity to TRAIL varied between the three cell lines, UMSCC11B was the most

sensitive. IC_{50} values were 0.7764, 2.821 and 110.3 ng/ml for UMSCC11B, H357 and UMSCC22B respectively.

In summary, the data showed that three out of the nine cell lines tested were Smac59 sensitive and TRAIL resistant, while six out of the nine cell lines were TRAIL sensitive and Smac59 resistant. Most of the sensitive cells were shown to be highly sensitive to the respective drug as well as to have a dose dependent pattern of sensitivity. For individual cell lines, it was interesting to see that the Smac59 sensitive cell lines were TRAIL resistant and vice versa. None of the cells tested was found to be sensitive or resistant to both drugs. Therefore, the mechanism of resistant/sensitivity of each cell line to these drugs was further investigated.

3.1.2 Sensitivity of HNSCC cell lines to combined TRAIL and Smac59 treatment

The effect of the combined treatment with both drugs was investigated to determine whether they could synergize to induce cytotoxicity. The HNSCC cell line panel was seeded in 96 well plates and after 24 hours of incubation at 37ºC and 5% CO_2 they were treated with TRAIL (50 ng/ml), Smac59 (28.33 nM) or both. The cells were incubated with the drugs for 72 hours and the cell viability was tested with MTT assay.

The results shown in Figure 3.4 confirmed the previous observations and indicated that the tested cells showed similar pattern of sensitivity to Smac59 and TRAIL. Smac59 and TRAIL in combination effectively inhibited cell viability of all the tested HNSCC cell lines. When Smac59 and TRAIL were combined, the reduction in the cell viability observed in the Smac59 sensitive cells was similar to the levels obtained with single Smac59 treatment, indicating that TRAIL did not increase the cytotoxicity of Smac59; the difference between the effect of Smac59 alone and the combination treatment was not statistically significance ($P > 0.05$). Conversely, the reduction in cell viability observed in the TRAIL sensitive cells was higher with the combination treatment than single TRAIL treatment; the difference in the response between single TRAIL treatment and combination treatment with

Smac59 was statistically significant ($P < 0.05$). This indicated that adding Smac59 to TRAIL enhanced TRAIL cytotoxic effect in TRAIL sensitive cells.

3.1.3 Sensitisation of TRAIL resistant cell lines to TRAIL using Smac59

The previous experiments suggested that adding Smac59 to TRAIL enhanced the cytotoxic effect of TRAIL in the TRAIL sensitive cell lines. Whether Smac59 enhanced TRAIL cytotoxic effect in TRAIL resistant cell lines was difficult to measure because of high susceptibility to Smac59 in TRAIL resistant cells. In order to test whether Smac59 could sensitise the TRAIL resistant cells to TRAIL, it was necessary to use a non toxic concentration of Smac59.

Smac59 sensitive cell lines, HSC3, HSC3M3 and HN5 were treated with either TRAIL (50 ng/ml), Smac59 (0.025 nM) or both in combination and cell viability was measured with MTT assay. As shown in Figure 3.5 (A and B), in HSC3 and HSC3M3 cell lines, adding non toxic concentrations of Smac59 to TRAIL resulted in 20-25% reduced cell viability than TRAIL alone, which was a statistically significant change (p value <0.01). This was not the case for HN5 cell line (Figure 3.5 C); the combined effect of both TRAIL and Smac59 was not significantly different than the effect of TRAIL alone for this cell line.

Table 3.1 Characteristic features of HNSCC cell lines

	HSC3	HSC3M3	HN5	HN30	H357	UMSCC74A	UMSCC74B	UMSCC11B	UMSCC22B
Age	63	63	73	Unknown	74	N/A	N/A	65	59
Sex	M	M	M	Unknown	M	N/A	N/A	M	F
Origin	Cervical lymph node/tongue carcinoma	Metastatic from HSC3 in mice	Tongue	Pharynx	Tongue	Base of tongue	Tongue	Hypopharynx	Lymph node metastasis/ hypopharynx
Tumour differentiation	Poorly diff.	Poorly diff.	Mod. diff.	N/A	Well diff	N/A	N/A	N/A	N/A
TNM	N/A	N/A	T2 N0M0	N/A	T1N0M0	N/A	N/A	T2N2aM0	T2N1M0
Stage	N/A	N/A	II	N/A	I	N/A	N/A	V	III
Prior therapy	N/A	N/A	Chemotherapy/ radiotherapy	None	None	None	Chemotherapy	Chemotherapy	None
P53 status	Mutant	Mutant	Mutant	Wild type	Mutant	Wild type	Wild type	Mutant	Mutant
Ras status	Mutant	Mutant	N/A	N/A	Mutant	N/A	N/A	N/A	N/A
HPV status	Negative	Negative	N/A	Negative	Negative	N/A	N/A	N/A	N/A

Abbreviations: M, male; F, female and N/A, not available.

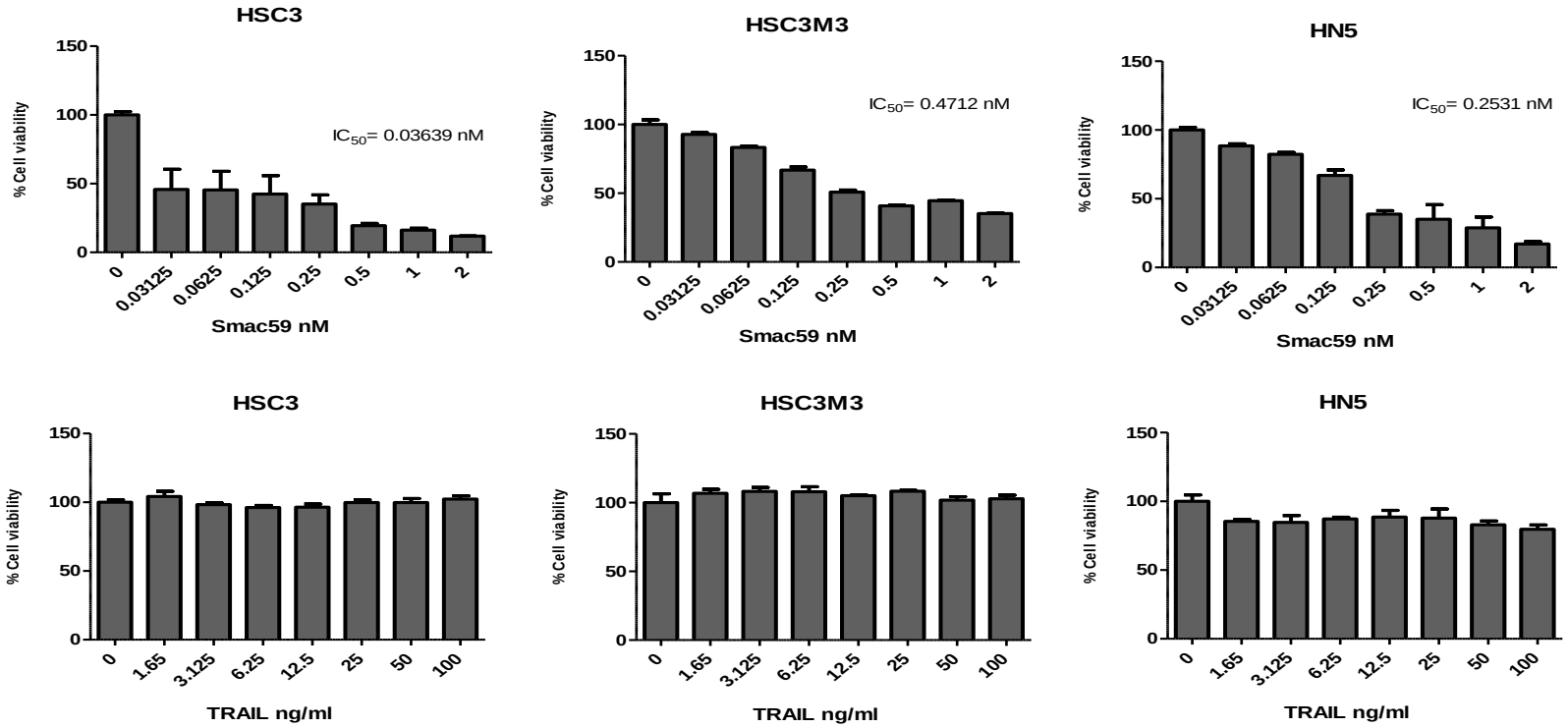


Figure 3.1 Effect of Smac59 and TRAIL on HSC3, HSC3M3 and HN5 cell lines at various concentrations

HSC3, HSC3M3 and HN5 cell lines were seeded in 96 well plates and treated with Smac59 (2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125nM) and TRAIL (100, 50, 25, 12.5, 6.25, 3.125 and 1.65ng/ml). Cells were incubated at 37ºC and 5%CO_2 for 72 hours then the cell viability was measured using MTT assay. The results are shown as the percentage of viable cells after treatment with respect to untreated viable cells. All experiments were performed in triplicate, error bars indicate SD.

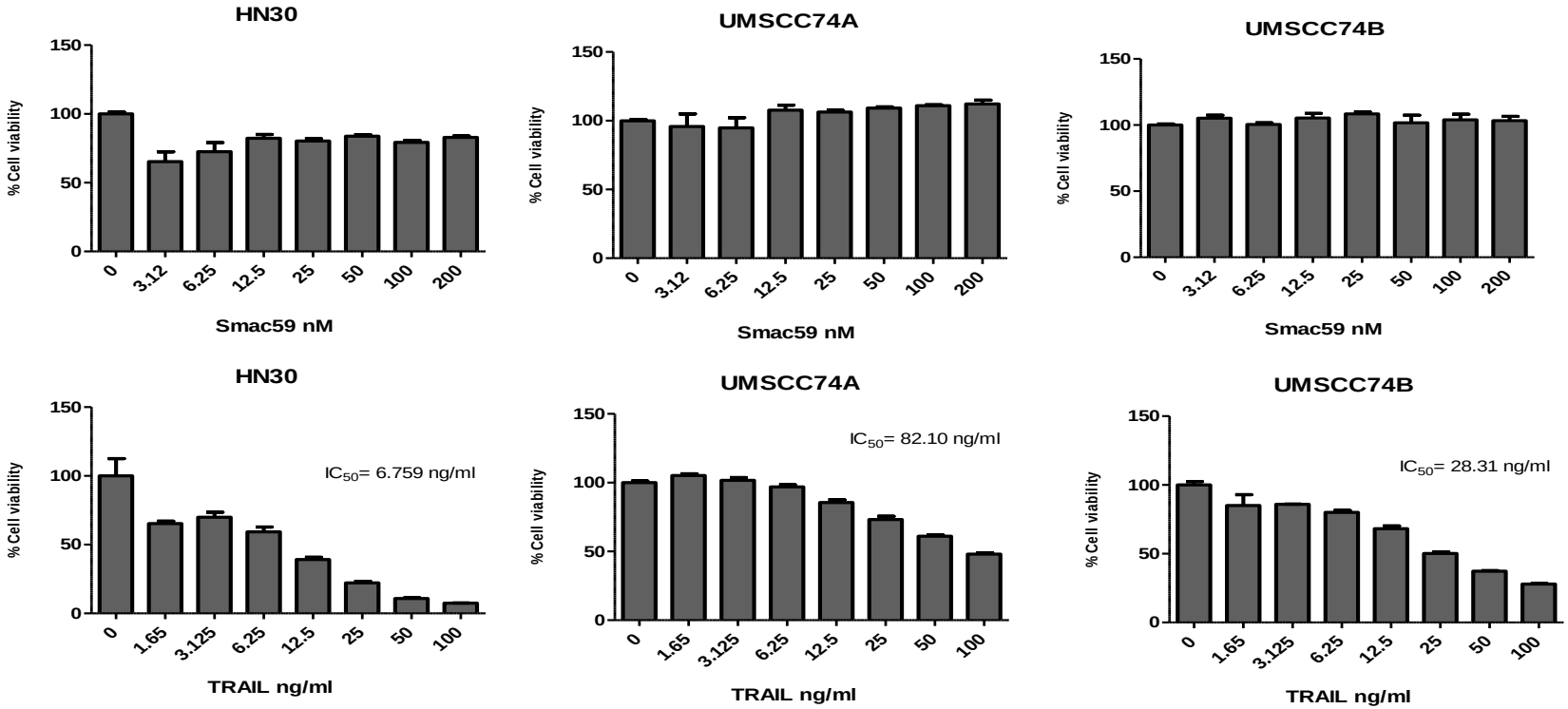


Figure 3.2 Effect of Smac59 and TRAIL on HN30, UMSCC74A and UMSCC74B cell lines at various concentrations

HN30, UMSCC74A and UMSCC74B cell lines were seeded in 96 well plates and treated with Smac59 (200, 100, 50, 25, 12.5, 6.25, 3.12nM) and TRAIL (100, 50, 25, 12.5, 6.25, 3.125 and 1.6 ng/ml). Cells were incubated at 37ºC and 5%CO_2 for 72 hours then the cell viability was measured using MTT assay. The results are shown as the percentage of viable cells after treatment with respect to untreated viable cells. All experiments were performed in triplicate, error bars indicate SD.

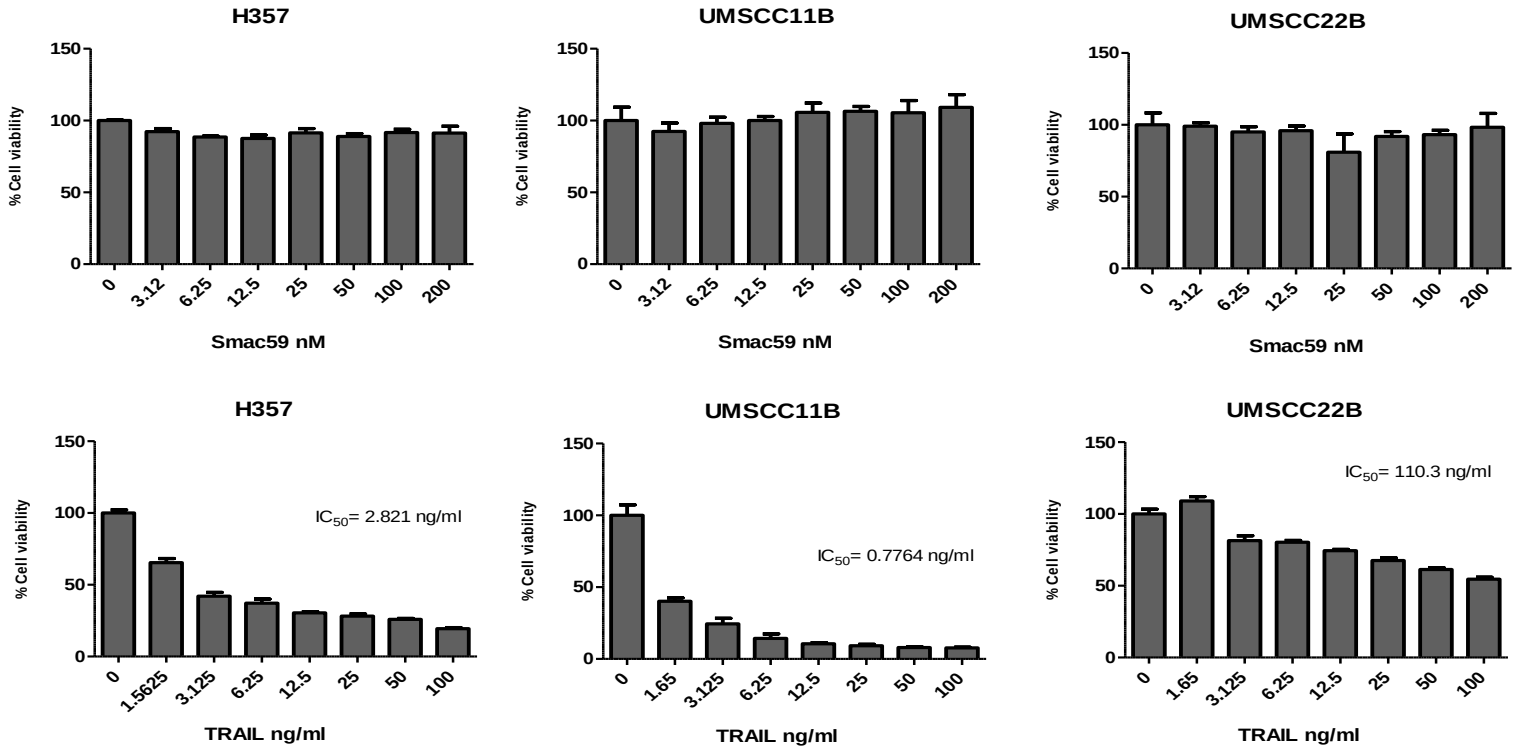


Figure 3.3 Effect of Smac59 and TRAIL titration on H357, UMSCC11B and UMSCC22B cell lines at various concentrations

H357, UMSCC11B and UMSCC22B cell lines were seeded in 96 well plates and treated with Smac59 (200, 100, 50, 25, 12.5, 6.25, 3.12nM) and TRAIL (100, 50, 25, 12.5, 6.25, 3.125 and 1.65ng/ml).). Cells were incubated at 37ºC and 5%CO_2 for 72 hours then the cell viability was measured using MTT assay. The results are shown as the percentage of viable cells after treatment with respect to untreated viable cells. All experiments were performed in triplicate, error bars indicate SD.

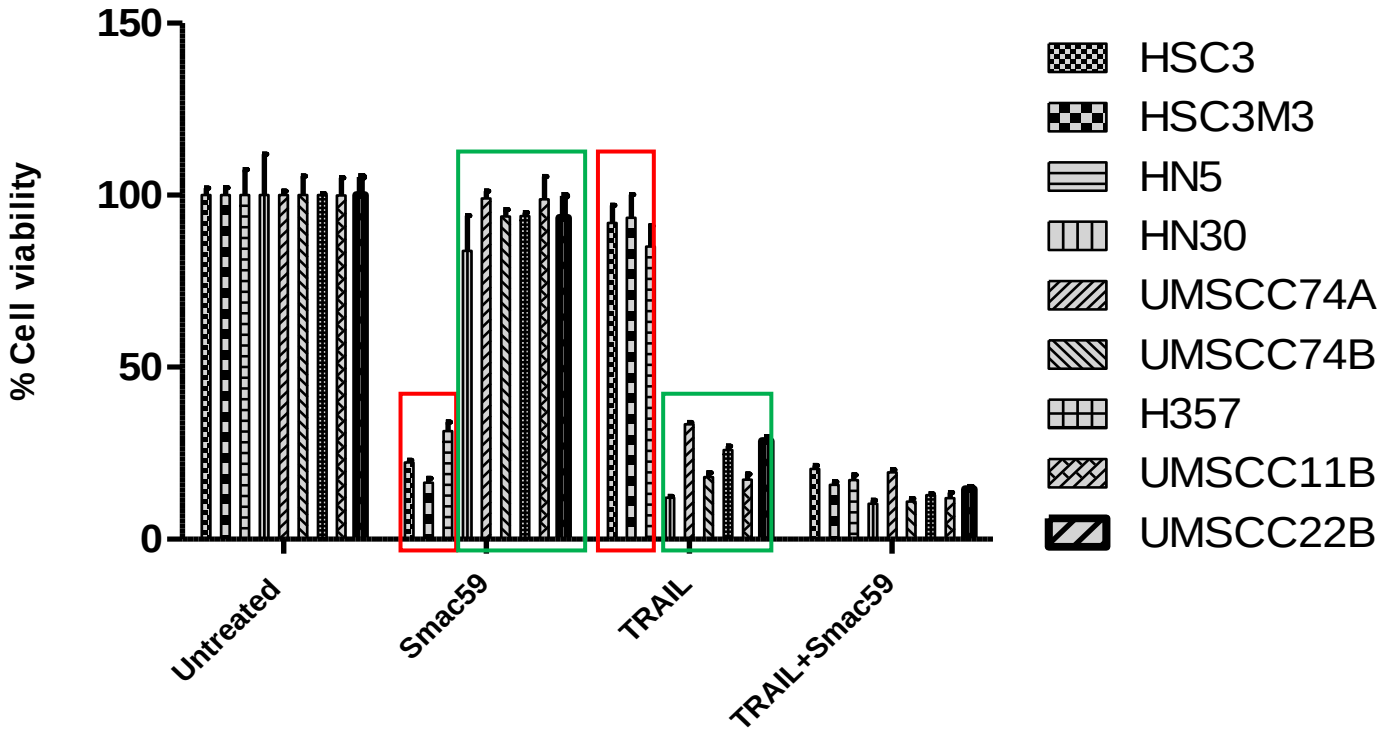


Figure 3.4 Effect of combined TRAIL and Smac59 treatment on HNSCC cell lines

Nine HNSCC cell lines were seeded in 96 well plates and treated with TRAIL (50ng/ml), Smac59 (28.33nM), or combination. Cells were incubated at 37ºC and 5%CO_2 for 72hours and the cell viability was measured using the MTT assay. The results are shown as the percentage of the viable cells after treatment with respect to viable untreated treated cells. All experiments were performed in triplicate, error bars indicate SD. Red rectangle encloses Smac59 sensitive cells and green rectangle encloses TRAIL sensitive cell lines.

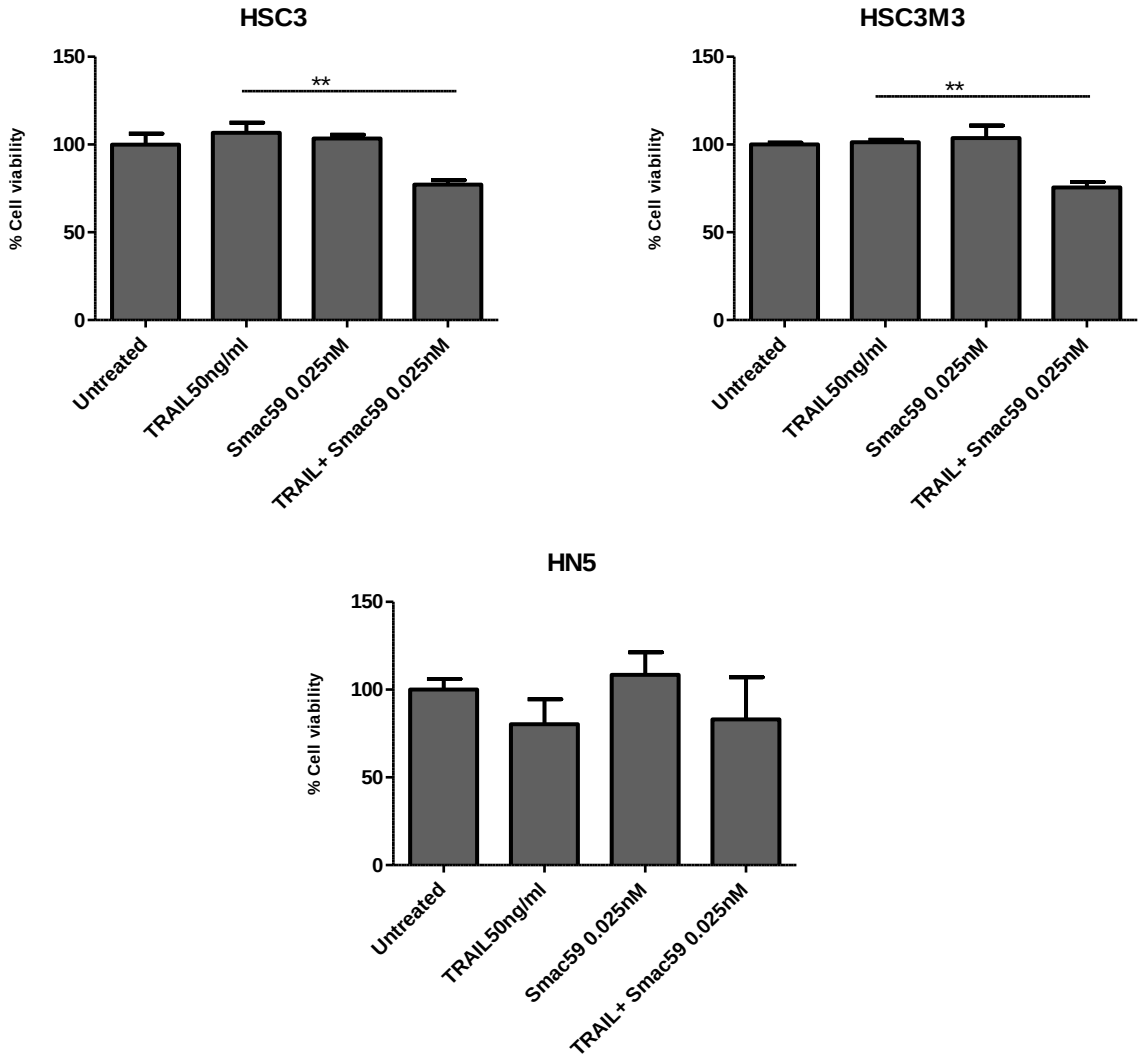


Figure 3.5 TRAIL sensitisation using Smac59 in HNSCC cell lines

HSC3, HSC3M3 and HN5 cell lines were seeded in 96 well plates and treated with TRAIL (50ng/ml) and Smac59 (0.025nM) or both combined for 72 hours. Cell viability was measured using MTT assay. The results are shown as the percentage of the viable cells after treatment with respect to untreated viable cells. All experiments were performed in triplicate, error bars indicate SD.

* indicates a significant difference between the tested groups indicated (unpaired two tailed t-test *p* value< 0.001).

3.1.4 Sensitivity of normal human keratinocytes to TRAIL and Smac59

As TRAIL and Smac59 have a clear cytotoxic effect on HNSCC cell lines, they might be future anticancer agents in head and neck carcinoma. However, one of the main limitations for most anticancer drugs is the non-specific cytotoxic effect on normal cells. To test the cytotoxic effect of TRAIL and Smac59 on normal epithelium, human oral keratinocytes (NHK) were seeded in 6 well plates, along with HSC3M3 and SCC11B cell lines. Cells were treated in duplicates with either TRAIL (50ng/ml), Smac59 (50nM) or left untreated. HSC3M3 and SCC11B were used as controls for sensitivity to drugs. The cell viability was tested using phase contrast microscope (to investigate changes in cell morphology) as well as using MTT assay.

The results shown in Figure 3.6A indicated that NHK treated with TRAIL showed signs of apoptosis as indicated by, membrane blebbing, cytosolic condensation and breakdown of nuclear DNA. The Smac59 treated NHK showed no difference from untreated control cells. These observations indicated that TRAIL was cytotoxic to normal head and neck cells while Smac59 was not. Drug activity was confirmed by positive control treatment of HSC3M3 and UMSCC11B cells. This killing of normal cells was further confirmed by measuring cell viability with MTT assay and the results are shown in Figure 3.6 B.

For further investigation of TRAIL and Smac59 effect on a non-tumourigenic cell line, this experiment was repeated using HaCat spontaneously immortalised non-tumourigenic keratinocyte. HaCat cells were sensitive to TRAIL and resistant to Smac59 as shown in Figure 3.7. Collectively, we show that TRAIL was effective in killing most of our cancer cell lines as well as normal and non-transformed immortalised cell lines.

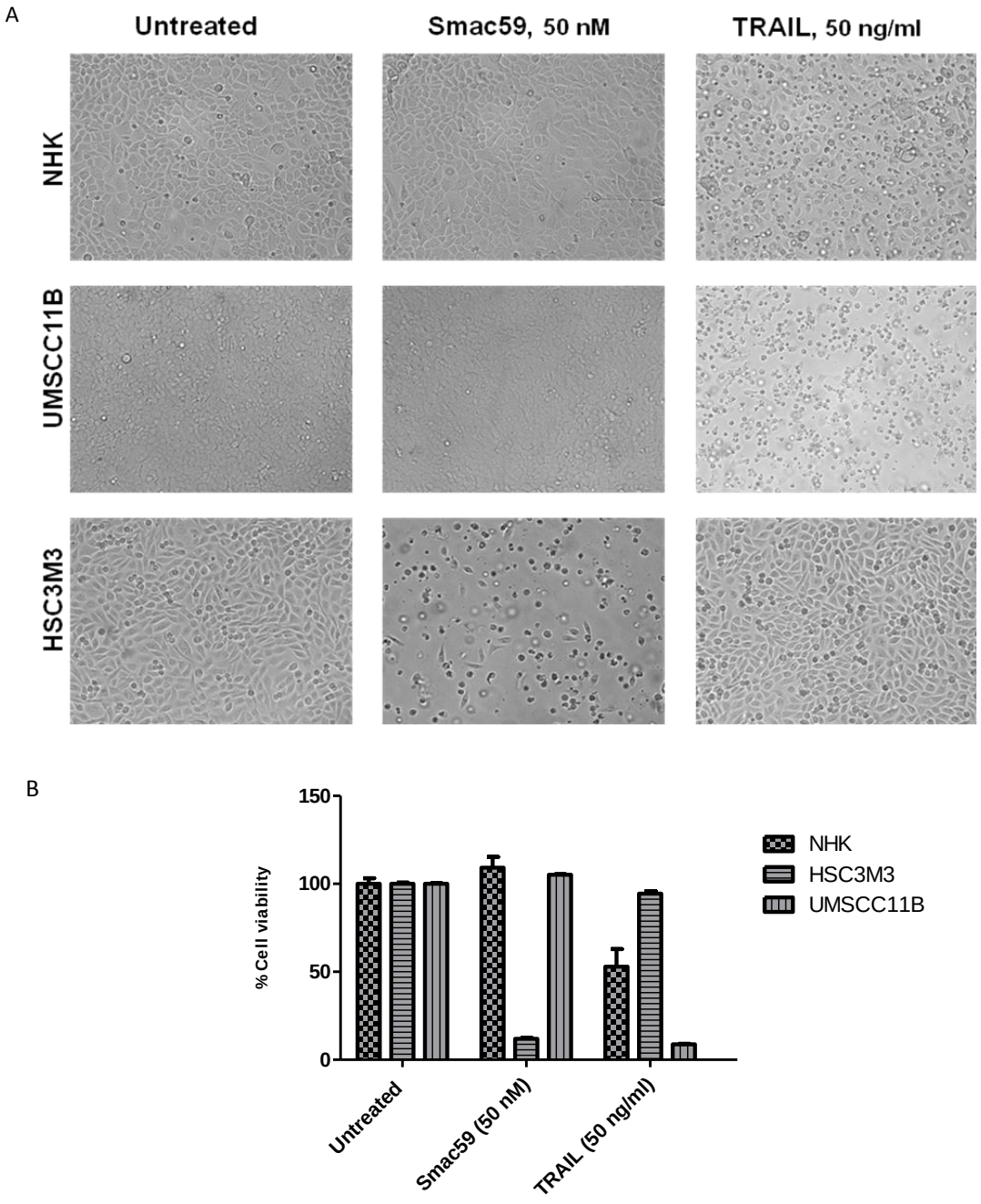


Figure 3.6 Sensitivity of Normal Human Keratinocytes (NHK) to TRAIL and Smac59

A: Normal human keratinocytes, HSC3M3 and UMSCC11B were seeded in 6 well plate and treated with TRAIL (50ng/ml) or Smac59 (50nM). Cytotoxic effect was determined by apoptotic morphological features using phase contrast microscopy. B: Normal human keratinocytes, HSC3M3 and UMSCC11B were seeded in 96 well plates and treated with TRAIL (50ng/ml) or Smac59 (50nM). Cell viability was determined by MTT assay, the results are shown as percentage of viable cells with respect to viable untreated cells. All experiments were performed in triplicate. Error bars indicates SD.

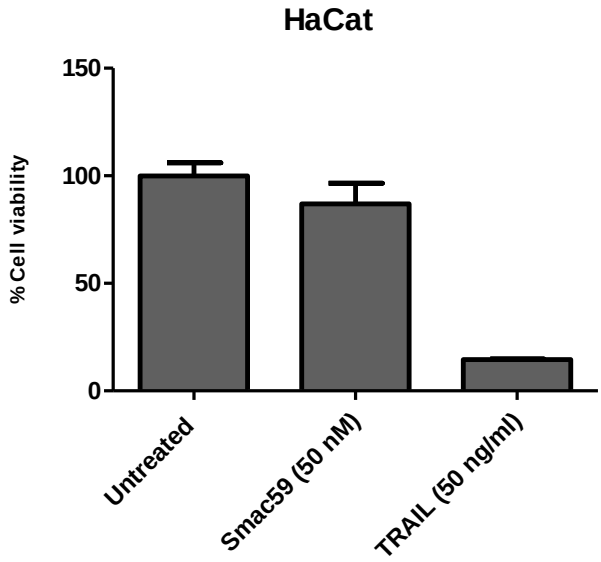


Figure 3.7 Sensitivity of HaCat cells to TRAIL and Smac59

HaCat cells were seeded in 96 well plates and treated with TRAIL (50ng/ml) and Smac59 (50nM) for 72 hours. Cell viability was measured using MTT assay. The results are shown as the percentage of the viable cells after treatment with respect to viable untreated cells. All experiments were performed in triplicate. Error bars indicate SD.

In summary, the cytotoxic effect of recombinant IZ-TRAIL and Smac59 was investigated using a panel of HNSCC cell lines, normal oral keratinocytes and spontaneously transformed keratinocyte cell lines. The data obtained suggested:

- Both drugs were effective in killing cancer cells even at very low concentrations when the cells are inherently sensitive. Six cell lines were TRAIL sensitive and three cell lines were Smac59 sensitive. Combined treatment was effective in killing all the cell lines tested.
- Smac59 was able to sensitise the TRAIL resistant cells to TRAIL. However, single drug treatment was efficient in killing the sensitive HNSCC cell lines.
- Normal primary or immortalised cells were sensitive to TRAIL but not to Smac59 treatment.

Predicting response of HNSCC cell lines to TRAIL and Smac mimetics will enable to achieve maximum efficiency of each drug. The next chapter will focus on the identification of molecular mechanisms underlying the response of HNSCC cell lines to TRAIL and Smac59.

4 Results II

Identification of biomarkers to predict response of HNSCC cell lines to TRAIL and Smac59

TRAIL induces cell death by activation of apoptosis pathways following the binding to its corresponding receptors, TRAIL-R1 (DR4) or TRAIL-R2 (DR5). Smac mimetics induce cell death by inducing the activation of procaspase-3 and promote the enzymatic activity of mature caspase-3 through the binding to inhibitor of apoptosis proteins (IAPs) (Chai et al., 2000). In this chapter, different proteins involved in the apoptotic pathways activated by TRAIL and Smac59 will be investigated to identify the possible predictors for the sensitivity/resistant pattern which were observed in HNSCC cell lines.

4.1 Expression analysis of target proteins

Expression of XIAP, cIAP-1, cIAP-2, Bcl-2, Mcl-1, Smac/DIABLO, Caspase-8, Caspase-3, FLIP and EGFR proteins were analysed. Nine HNSCC cell lines; three Smac59 sensitive (HSC3, HSC3M3 and HN5) and six TRAIL sensitive (HN30, H357, UMSCC74A, UMSCC74B, UMSCC11B and UMSCC22B), normal human oral keratinocyte (NHK) and immortalised keratinocyte HaCat cells were seeded in 10cm dishes and incubated at 37ºC and 5% CO_2 for 48-72 hours depending on the cell line. When the cells reached 80-90% confluence, they were washed with cold PBS and then 500µl of lysis buffer was added and the cell lysates were collected using sterile cell scrapers. Protein analysis was performed using western blotting.

In order to obtain approximate values for expression level of different proteins, the bands obtained from western blot analysis were quantified using ImagJ program. The quantification was performed for the bands of the protein of interest normalized to β-actin used as internal loading control. The data were plotted on excel worksheets to produce graphs representing protein expression levels in different cell lines.

4.1.1 Inhibitors of apoptosis proteins (IAPs)

Cancer cells acquire resistance to apoptosis by upregulating multiple pro-survival factors. The inhibitors of apoptosis proteins are a family of eight members that potently suppresses apoptosis triggered by a large variety of apoptotic stimuli. All members can block the caspase cascade but only some of them directly interact with caspases (Hunter et al., 2007).

4.1.1.1 X-linked inhibitor of apoptosis protein (XIAP)

XIAP is the most potent inhibitor of apoptosis of all the inhibitor of apoptosis proteins family (IAPs). XIAP effectively inhibits both intrinsic and extrinsic apoptosis pathways by binding and inhibiting the initiator caspase-9 and the effector caspase-3 and -7, whose activity is crucial for apoptosis execution (Deveraux et al., 1997). XIAP is over-expressed in many cancer cell lines and tumours but not in normal cells, and a high level of XIAP results in apoptosis-resistance of cancer cells to a wide variety of therapeutic agents (Dai et al., 2009a). XIAP expression levels could affect TRAIL/Smac59 sensitivity in HNSCC cell lines. In this study we investigated the expression levels of XIAP protein in both cancer and normal cell lines and correlated this with the sensitivity/resistant to TRAIL and Smac59.

The results shown in Figure 4.1 indicated that all HNSCC cell lines tested as well as NHK cells expressed XIAP. HaCat cells showed undetectable XIAP expression. With the exception of H357 cell line, HNSCC cell lines expressed more XIAP than normal NHK. This data indicated that XIAP is overexpressed in HNSCC cell lines. No correlation between the XIAP expression levels and TRAIL or Smac59 sensitivity of the tested cell lines was observed.

4.1.1.2 Cellular inhibitor of apoptosis proteins (cIAP-1 and cIAP-2)

cIAP-1 and cIAP-2 are members of IAPs family. They inhibit death receptor-mediated apoptosis by regulating receptor mediated signaling pathways upstream of mitochondria through the interaction with TNF receptor associated

factor 1 and 2, TRAF1 and TRAF2, (Guicciardi et al., 2011). cIAP-1 is known to be highly expressed in cancer cells however cIAP-2 is present in low abundance in the cells (LaCasse et al., 2008).

The results shown in Figure 4.2 indicated that cIAP-1 was expressed by all HNSCC cell lines and normal cell lines tested. No major differences in cIAP-1 expression between normal and cancer cell lines were observed. No correlation between level of cIAP-1 in HNSCC cell lines and their sensitivity/resistant to TRAIL or Smac59 was detected.

With regard to cIAP-2 expression, the results shown in Figure 4.3 indicated that five (HSC3, HN30, H357, UMSCC11B and UMSCC22B) of the nine HNSCC cell lines tested expressed cIAP-2 at different levels; HN30 and H357 cell lines expressed the highest level. cIAP-2 expression in HSC3M3, HN5, UMSCC74A and UMSCC74B was undetectable. cIAP-2 expression in HaCat cells was high, but NHK cells showed low cIAP-2 expression level. With the exception of UMSCC74A and UMSCC74B, cIAP-2 expression levels showed correlation with TRAIL sensitivity in HNSCC cell lines; TRAIL sensitive cell lines showed higher expression levels of cIAP-2 than TRAIL resistant cell lines.

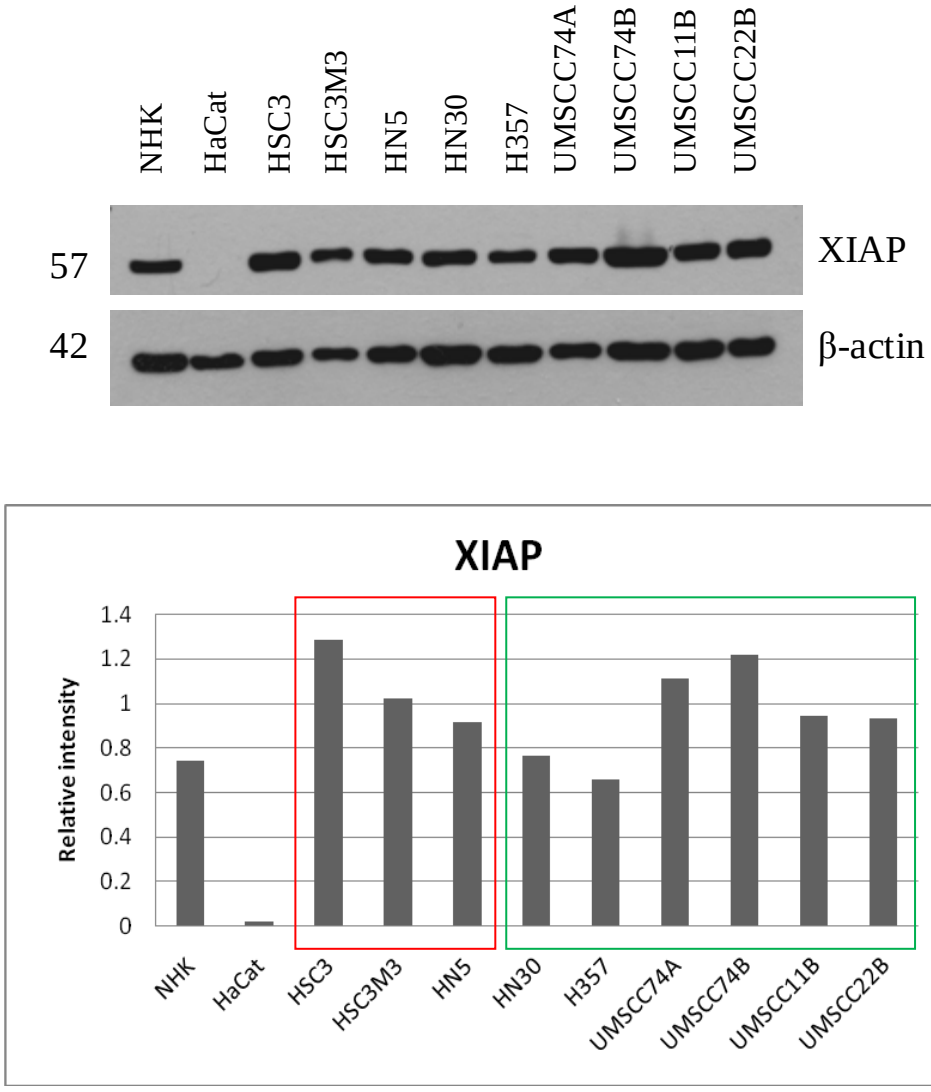
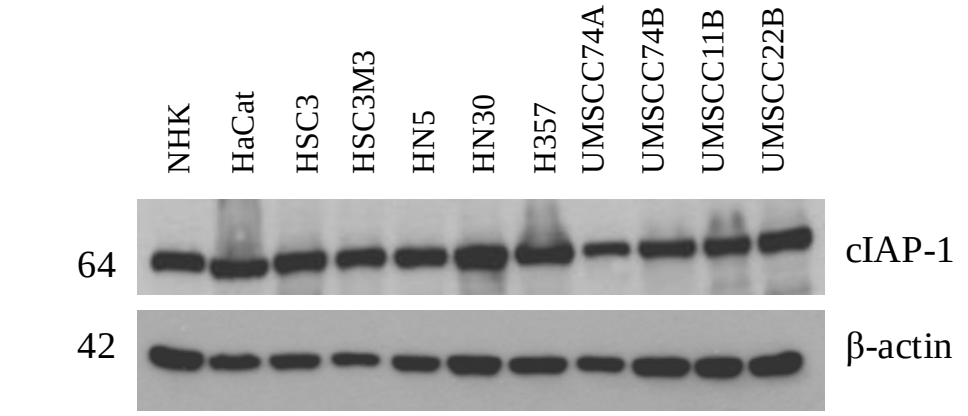


Figure 4.1 XIAP protein expression in HNSCC cell lines

Nine HNSCC cell lines, NHK and HaCat cells (untreated) were lysed and subjected to western blot analysis for endogenous XIAP protein expression (top). The intensity of the bands from the western blotting was quantified in relation to β-actin level using the ImagJ program. Red rectangle encloses the Smac59 sensitive (TRAIL resistant) cell lines and the green rectangle encloses the Smac59 resistant (TRAIL sensitive) cell lines.



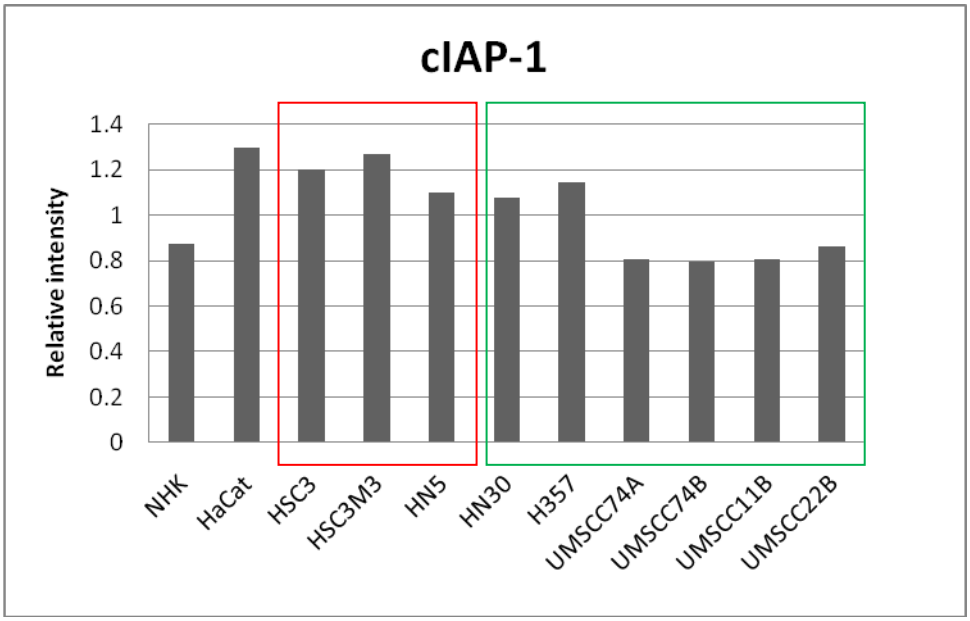
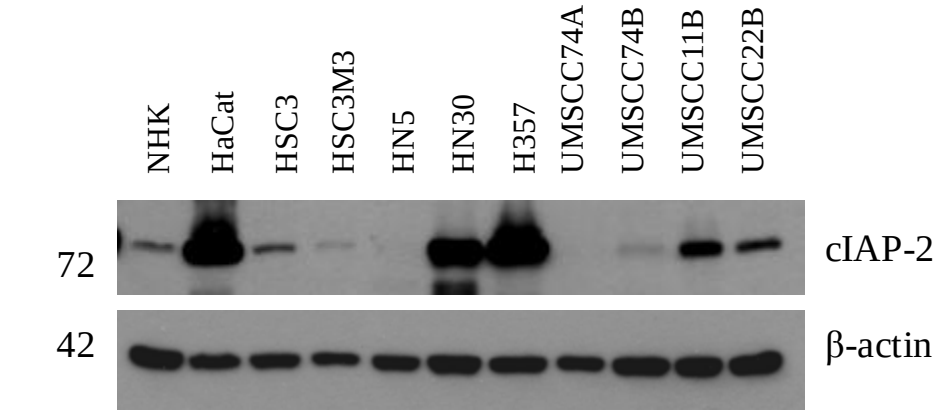


Figure 4.2 cIAP-1 protein expression in HNSCC cell lines

Nine HNSCC cell lines, NHK and HaCat cells (untreated) were lysed and subjected to western blot analysis for endogenous cIAP-1 protein expression (top). The intensity of the bands from the western blotting was quantified in relation to β-actin level using the ImagJ program. Red rectangle encloses the Smac59 sensitive (TRAIL resistant) cell lines and the green rectangle encloses the Smac59 resistant (TRAIL sensitive) cell lines.



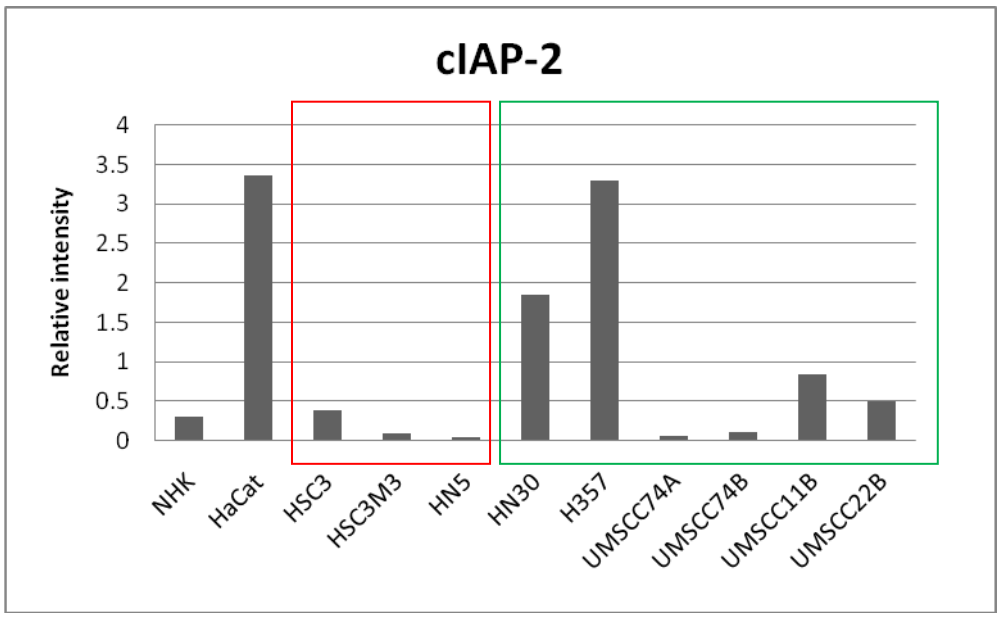


Figure 4.3 cIAP-2 protein expression in HNSCC cell lines

Nine HNSCC cell lines, NHK and HaCat cells (untreated) were lysed and subjected to western blot analysis for endogenous cIAP-2 protein expression (top). The intensity of the bands from the western blotting was quantified in relation to β-actin level using the ImagJ program. Red rectangle encloses the Smac59 sensitive (TRAIL resistant) cell lines and the green rectangle encloses the Smac59 resistant (TRAIL sensitive) cell lines.

4.1.2 Smac/DIABLO

Smac/DIABLO (second mitochondria-derived activator of caspases, direct IAP-binding protein with low PI) is a 25 kDa mitochondrial protein that promotes apoptosis through its ability to antagonize IAP-mediated caspase inhibition once released into the cytoplasm following intrinsic apoptosis pathway activation. It functions by binding to IAPs which results in releasing their inhibitory effects on caspases (Du et al., 2000). Therefore sensitivity of cancer cells to different therapeutics could be influenced by the amount of Smac/DIABLO available to antagonise the inhibitory effect of IAPs.

The results shown in Figure 4.4 indicated that all HNSCC cell lines as well as NHK and HaCat cell lines expressed endogenous Smac/DIABLO at different levels with NHK showing the lowest level of expression. Smac59 sensitive (TRAIL resistant) cell lines (HSC3 and HN5) expressed more Smac/DIABLO than Smac59 resistant (TRAIL sensitive) cell lines which indicated that a correlation between the level of expression of Smac/DIABLO and Smac59 sensitivity TRAIL resistant in the tested cells was identified.

4.1.3 Bcl-2 family

Bcl-2 family members are divided into three categories of protein according to their structure and function and are defined as: anti-apoptotic, multi-domain pro-apoptotic and BH3-only pro-apoptotic members. As mentioned in the introduction, Bcl-2 family members are responsible for determining whether or not the apoptosome complex can assemble and consequently execute apoptosis.

4.1.3.1 Bcl-2 protein

Bcl-2 is an anti-apoptotic member that potently inhibits apoptosis, mediated by inhibition of apoptosome formation. Endogenous bcl-2 protein level was investigated in HNSCC cell lines and in NHK and HaCat cells.

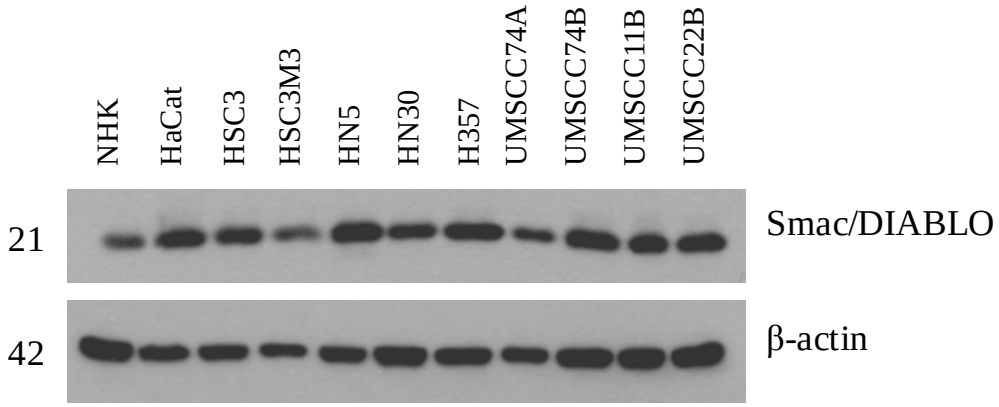
As shown in Figure 4.5, bcl-2 protein expression was undetectable in Smac59 sensitive (TRAIL resistant) cell lines HSC3 and HSC3M3. Conversely

Smac59 resistant (TRAIL sensitive) cell lines UMSCC74A, UMSCC74B, UMSCC11B and UMSCC22B, showed considerably high levels of bcl-2 protein expression. Neither NHK nor HaCat cell lines showed detectable bcl-2 expression. With the exception of HN5, HN30 and H357, bcl-2 expression pattern in HNSCC cell lines correlated with TRAIL and Smac59 sensitivity. Smac59 sensitive (TRAIL resistant) cell lines had lower bcl-2 levels than Smac59 resistant (TRAIL sensitive) cell lines.

4.1.3.2 MCL-1

Mcl-1 is an anti-apoptotic member of the bcl-2 family that promotes cell survival by inhibiting release of cytochrome c from the mitochondria. Mcl-1 has a short half life and plays a critical role in determining the fate of the cells. The level of Mcl-1 may be a useful predictive indicator for the treatment of various cancers (Michels et al., 2005)

In this study, Mcl-1expression was observed in all the nine cell lines tested and NHK and HaCat. Level of Mcl-1 expression was varied by cell line. HaCat cell line had the highest level of Mcl-1 expression of all tested cells. Mcl-1 expression was higher in Smac59 sensitive (TRAIL resistant) cell lines than in Smac59 resistant (TRAIL sensitive) cell lines (Figure 4.6) indicating that resistance to TRAIL could be associated with Mcl-1 level in the cells.



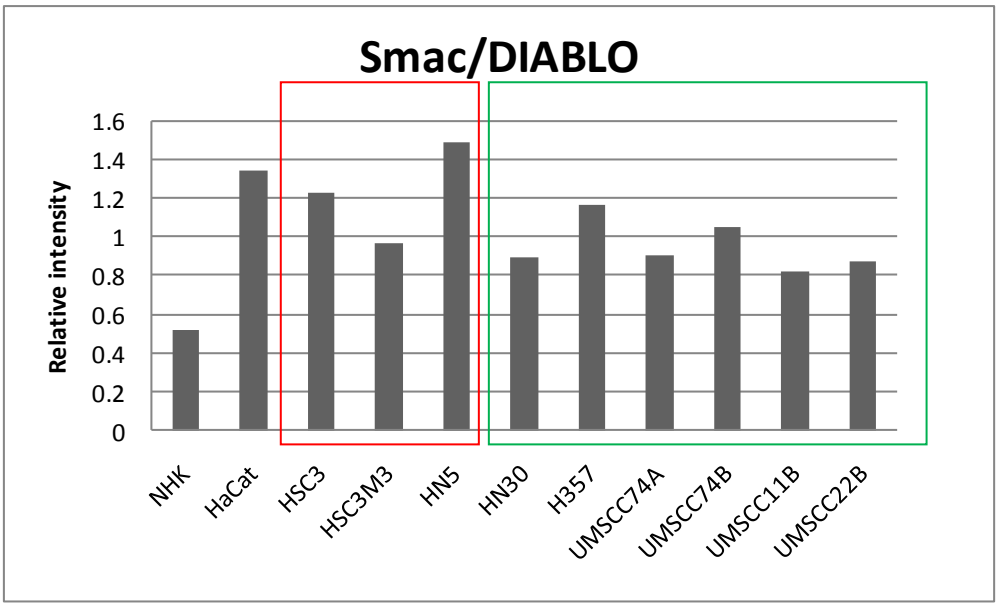


Figure 4.4 Smac/DIABLO protein expression in HNSCC cell lines

Nine HNSCC cell lines, NHK and HaCat cells (untreated) were lysed and subjected to western blot analysis for endogenous Smac/DIABLO protein expression (top). The intensity of the bands from the western blotting was quantified in relation to β-actin level using the ImagJ program. Red rectangle encloses the Smac59 sensitive (TRAIL resistant) cell lines and the green rectangle encloses the Smac59 resistant (TRAIL sensitive) cell lines.

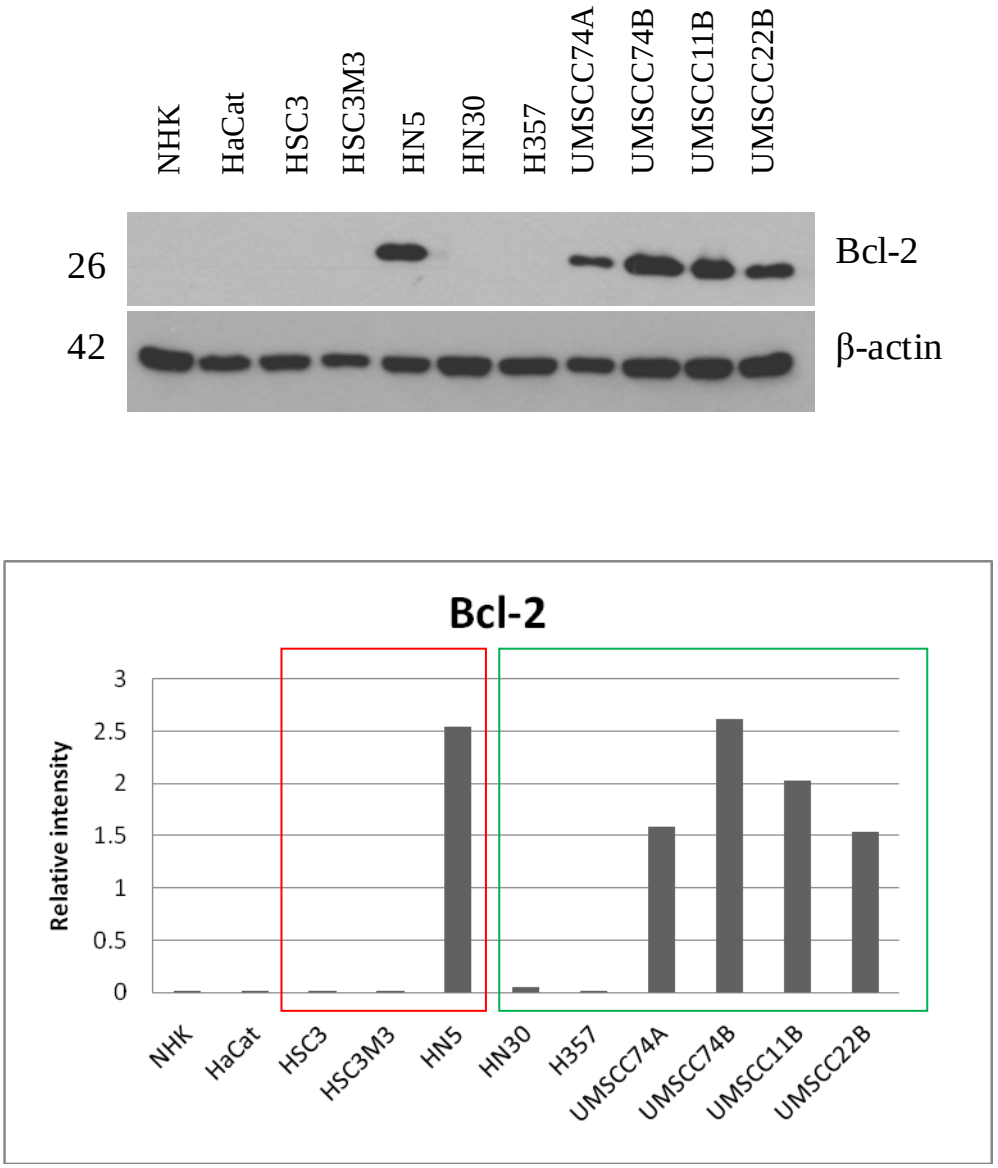


Figure 4.5 Bcl-2 protein expression in HNSCC cell lines

Nine HNSCC cell lines, NHK and HaCat cells (untreated) were lysed and subjected to western blot analysis for endogenous Bcl-2 protein expression (top). The intensity of the bands from the western blotting was quantified in relation to β-actin level using the ImagJ program. Red rectangle encloses the Smac59 sensitive (TRAIL resistant) cell lines and the green rectangle encloses the Smac59 resistant (TRAIL sensitive) cell lines.

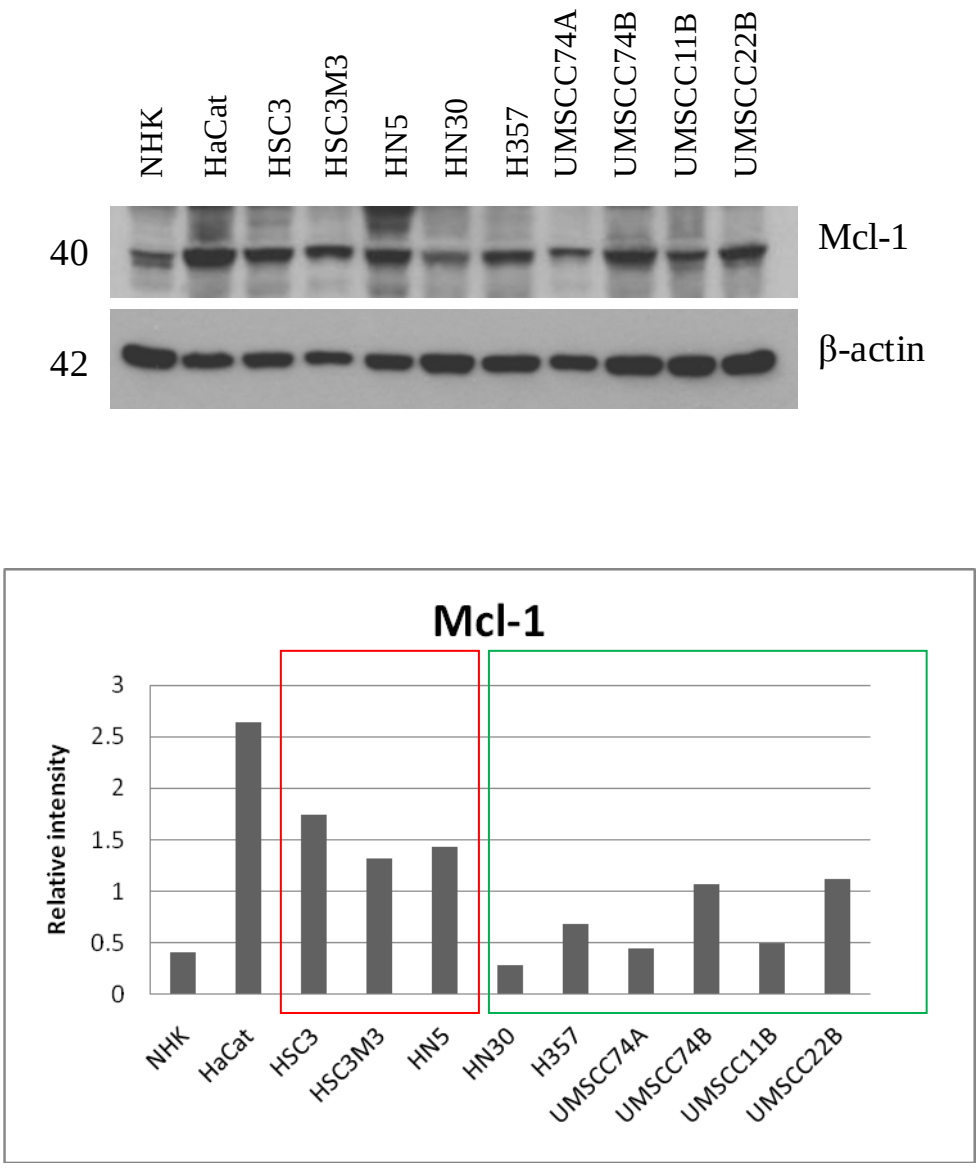


Figure 4.6 Mcl-1 protein expression in HNSCC cell lines

Nine HNSCC cell lines, NHK and HaCat cells (untreated) were lysed and subjected to western blot analysis for endogenous Mcl-1 protein expression (top). The intensity of the bands from the western blotting was quantified in relation to β-actin level using the ImagJ program. Red rectangle encloses the Smac59 sensitive (TRAIL resistant) cell lines and the green rectangle encloses the Smac59 resistant (TRAIL sensitive) cell lines.

4.1.4 Caspases

Initiation and execution of apoptosis relies on a complex network of cysteine proteases named caspases that exist in the cytoplasm as inactive pro-enzymes and are activated by proteolysis. Caspases involved in apoptosis are divided into two main groups: initiator and effector caspases. Caspase-8 and -10 initiate the extrinsic pathway. Caspase-9 is involved in the intrinsic pathway. Activation of this upstream caspases leads to activation of the effector caspase-3, -6 and -7 which are involved in the cleavage of specific proteins causing the characteristic apoptotic appearance (Lopez-Hernandez et al., 2003).

4.1.4.1 Caspase-8

Caspase-8 initiates the extrinsic pathway and it is activated upon binding of cell surface death receptors to their ligand. In type-I cells, activation of death receptors is sufficient to induce apoptosis while in type-II cells co-activation of the mitochondrial pathway is necessary to induce effector caspase activation. Activation of the intrinsic pathway is mediated via activated caspase-8 (Scaffidi et al., 1998).

The results in Figure 4.7 showed that Smac59 resistant (TRAIL sensitive) cell lines (HN30, H357, UMSCC74A, UMSCC74B, UMSCC11B and UMSCC22B) had a higher level of caspase-8 expression than Smac59 sensitive (TRAIL resistant) cell lines (HSC3, HSC3M3 and HN5). Caspase-8 expression was also detected in the NHK and HaCat cell lines. Casapase-8 level directly correlated with TRAIL sensitivity in HNSCC cell lines, suggesting that lack of caspase-8 may be the cause of HSC3, HSC3M3 and HN5 cell lines being resistant to TRAIL.

4.1.4.2 Caspase-3

Caspase-3 is an executioner caspase, its activation leads to cleavage of specific cellular proteins marking the irreversible cell death. Caspase-3 expression was investigated in HNSCC cell lines. The results shown in Figure 4.8 indicated that caspase-3 was expressed in all HNSCC cell lines as well as in NHK and HaCat

cell lines. No correlation was observed between caspase-3 levels and TRAIL or Smac59 sensitivity/resistant in HNSCC cell lines.

4.1.5 Cellular FLICE-inhibitory Protein (c-FLIP)

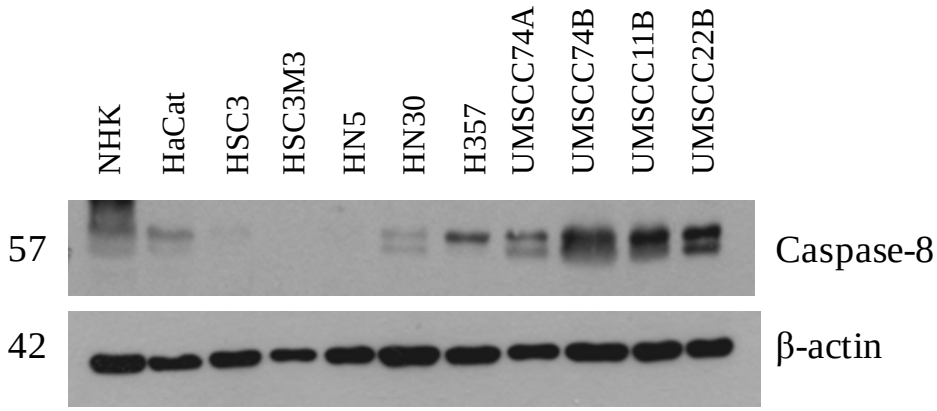
c-FLIP proteins exist as a long (c-$FLIP_L$) and a short (c-$FLIP_S$) splice variant. Both forms protect the cells from death receptor-mediated apoptosis by blocking pro-caspase-8 activation at the death inducing signalling complex (DISC) (Krueger et al., 2001).

The results shown in Figure 4.9 indicated that only the long (c-$FLIP_L$) variant was detected in the tested cell lines. Smac59 sensitive cell lines (HSC3 and HSC3M3) had a lower level of c-FLIP in comparison with TRAIL sensitive cell lines (HN30, H357, UMSCC22B, UMSCC11B, UMSCC74A and UMSCC74B). Interestingly, NHK and HaCat cells had a higher level of c-FLIP than HNSCC cell lines.

4.1.6 Epidermal growth factor receptor (EGFR)

EGFR is overexpressed in many solid cancers and has been shown to be involved in the development and progression of human malignancies. EGFR regulates many cellular function including cell growth, angiogenesis, inhibition of apoptosis, cell adhesion, cell motility and invasion (Lui and Grandis, 2002). Over-expression of EGFR in many cancers has been associated with resistance to treatment. In this study we investigated whether EGFR expression was associated with TRAIL and Smac59 sensitivity/resistance pattern in HNSCC cell lines.

The results shown in Figure 4.10 indicated that HSC3, HSC3M3, HN5, HN30 and H357 cell lines expressed EGFR at different levels while UMSCC (74A, 74B, 11B and 22B) showed no detectable EGFR expression. A direct association between EGFR expression and sensitivity to TRAIL and Smac59 was observed as Smac59 sensitive (TRAIL resistant) cell lines expressed higher levels of EGFR than Smac59 resistant (TRAIL sensitive) cell lines. NHK cells expressed EGFR protein while HaCat cells showed undetectable EGFR expression.



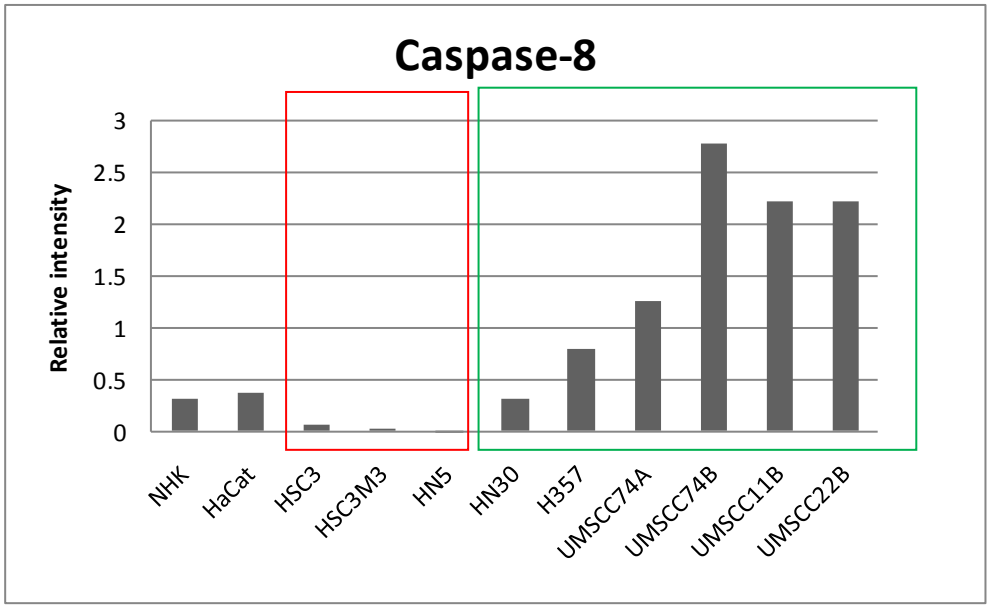
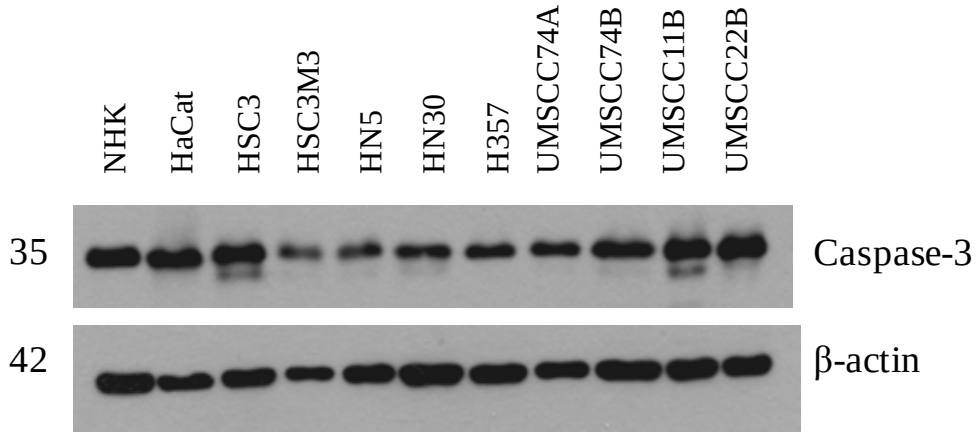


Figure 4.7 Caspase-8 protein expression in HNSCC cell lines

Nine HNSCC cell lines, NHK and HaCat cells (untreated) were lysed and subjected to western blot analysis for endogenous caspase-8 protein expression (top). The intensity of the bands from the western blotting was quantified in relation to β-actin level using the ImagJ program. Red rectangle encloses the Smac59 sensitive (TRAIL resistant) cell lines and the green rectangle encloses the Smac59 resistant (TRAIL sensitive) cell lines.



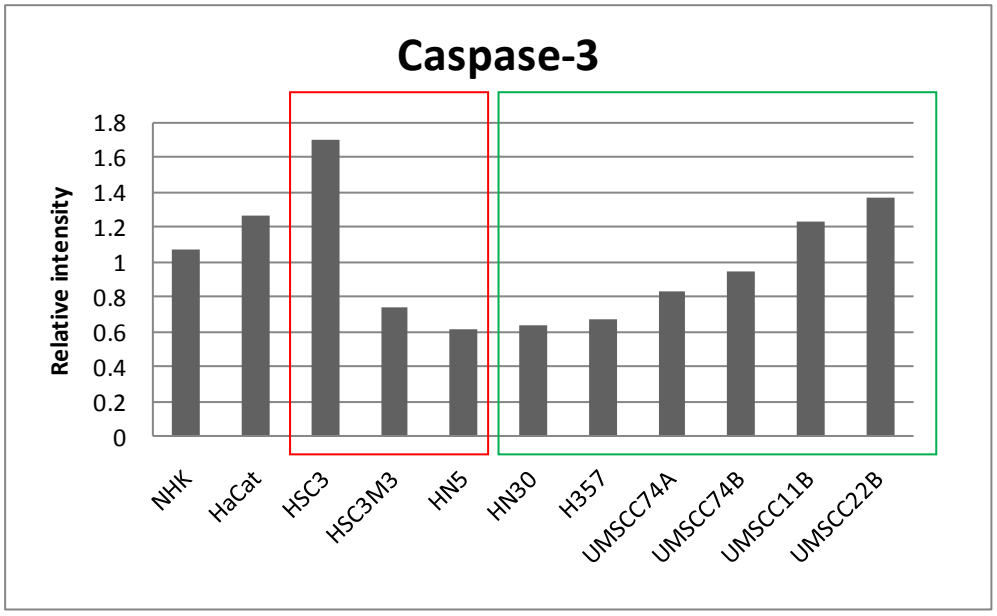
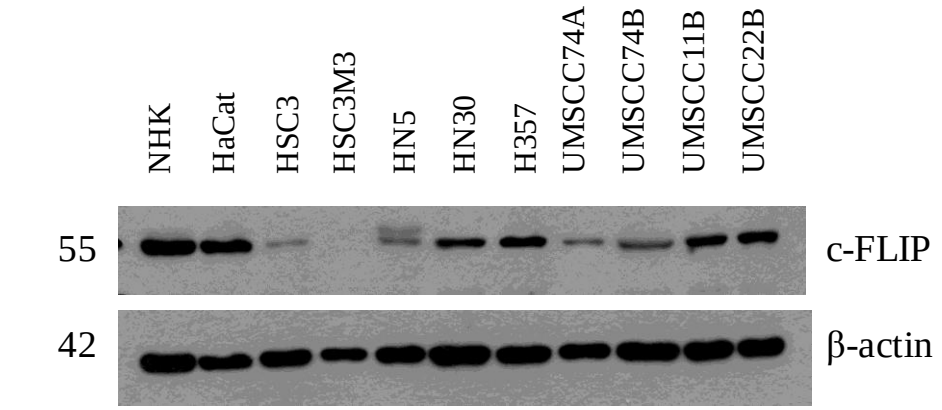


Figure 4.8 Caspase-3 protein expression in HNSCC cell lines

Nine HNSCC cell lines, NHK and HaCat cells (untreated) were lysed and subjected to western blot analysis for endogenous caspase-3 protein expression (top). The intensity of the bands from the western blotting was quantified in relation to β-actin level using the ImagJ program. Red rectangle encloses the Smac59 sensitive (TRAIL resistant) cell lines and the green rectangle encloses the Smac59 resistant (TRAIL sensitive) cell lines.



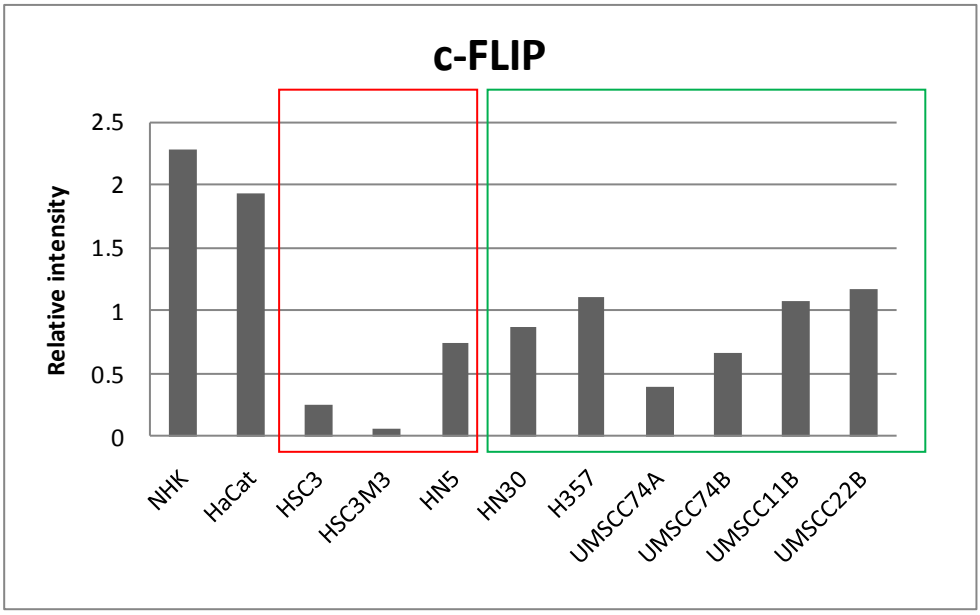
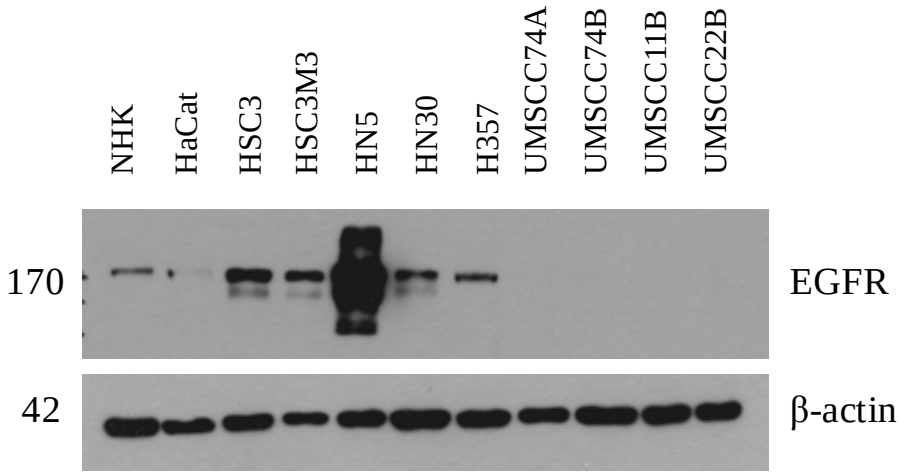


Figure 4.9 c-FLIP protein expression in HNSCC cell lines

Nine HNSCC cell lines, NHK and HaCat cells (untreated) were lysed and subjected to western blot analysis for endogenous FLIP protein expression (top). The intensity of the bands from the western blotting was quantified in relation to β-actin level using the ImagJ program. Red rectangle encloses the Smac59 sensitive (TRAIL resistant) cell lines and the green rectangle encloses the Smac59 resistant (TRAIL sensitive) cell lines.



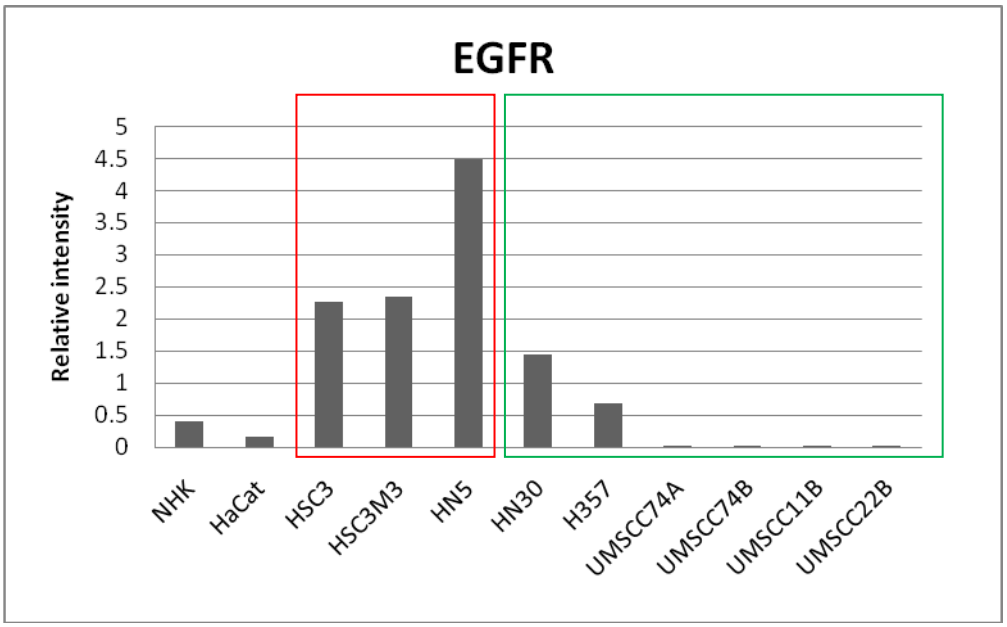


Figure 4.10 EGFR protein expression in HNSCC cell lines

Nine HNSCC cell lines, NHK and HaCat cells (untreated) were lysed and subjected to western blot analysis for endogenous EGFR protein expression (top). The intensity of the bands from the western blotting was quantified in relation to β-actin level using the ImagJ program. Red rectangle encloses the Smac59 sensitive (TRAIL resistant) cell lines and the green rectangle encloses the Smac59 resistant (TRAIL sensitive) cell lines.

4.2 Tumour necrosis factor alpha (TNF-α) and TRAIL/Smac59 sensitivity

Tumour necrosis factor alpha (TNF–α) is a pleiotropic ligand of tumour necrosis factor receptor 1 and 2 (TNFR1 and TNFR2) that can signal both cell survival and cell death. The pro-survival and the pro-death signalling aspects of TNF-α are mediated through two different protein complexes. Various human cancer cell lines undergo apoptosis upon Smac mimetic treatment without the need for exogenous pro-apoptotic stimuli. Smac mimetics were able to switch the pro-survival signal to cell death in certain cancer cells that secrete TNF-α (Petersen et al., 2007). The underlying mechanisms by which Smac mimetics induce cell death through TNF-α pathway remains unknown.

To investigate the role of TNF-α in TRAIL or Smac59 sensitivity in HNSCC cell lines, three Smac59 sensitive (TRAIL resistant) cell lines (HSC3, HSC3M3 and HN5) and three Smac59 resistant (TRAIL sensitive) (HN30, H357 and UMSCC11B) cell lines were analyzed for the level of TNF-α protein secreted in their growth media using ELISA. Equal numbers of cells were seeded in 12well plates in 1ml of media, incubated at 37ºC and 5% CO_2 for 24 hours then the medium was replaced with fresh medium (with or without serum). The cells were then incubated for 24 hours after which the medium was collected and stored at -70ºC. The level of TNF-α in the medium was measured using Human TNF-α CytoSetTM ELISA Kit.

This kit provides a recombinant h-TNF-α and a standard curve for TNF-α was generated to ensure linear correlation between colour intensities and TNF-α concentration and to obtain the curve equation used to calculate the TNF-α concentration in the test samples. Experiments were performed in duplicate and ELISA was performed in triplicate for each of duplicate samples.

The results in Figure 4.11 show the standard curve for TNF-α with the equation (A) and the level of secreted TNF-α in the test samples (B). Smac59 sensitive (TRAIL resistant) cell lines (HSC3 and HSC3M3) secreted a higher level

of TNF-α than Smac59 resistant (TRAIL sensitive) cell lines (HN30, H357 and UMSCC11B). The level of TNF-α in HN5 cell line, Smac59 sensitive, was not as high as other Smac sensitive cell lines. TNF-α secretion was tested in the presence and absence of serum in growth media. Presence or absence of serum had minimal effect on TNF-α secretion. It was concluded from these data that there is a direct correlation between the level of TNF-α secreted by HNSCC cell lines and their sensitivity to Smac59.

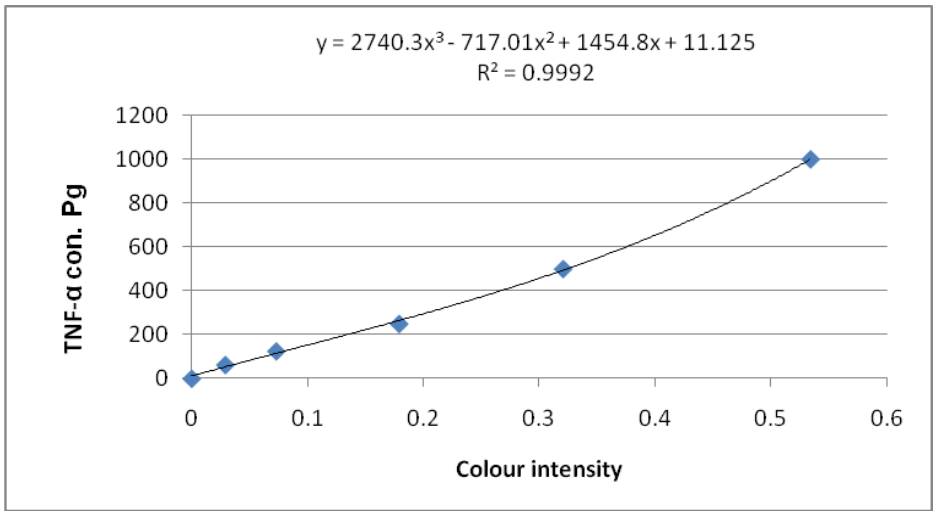
4.3 TRAIL receptors and TRAIL/Smac59 sensitivity.

TRAIL triggers apoptosis via interaction with TRAIL receptors in target cells. Five receptors bind TRAIL; TRAIL-R1 (DR4, TNSFRSF10a) and TRAIL-R2 (DR5, TNSFRSF10b) contain an extracellular ligand-binding domain and a cytoplasmic death domain that is required for activation of extrinsic apoptotic pathway after TRAIL binding. TRAIL-R3 (DcR1, TNFRSF10c), TRAIL-R4 (DcR2, TNFRSF10d) and circulating osteoprotegerin (OPG, TNFRSF11b) lack a functional death domain and are thought to be involved mainly in negatively regulating apoptosis by sequestering TRAIL or stimulating pro-survival signals (Kruyt, 2008). The ability of TRAIL to induced apoptosis in different cell lines could be affected by the number of its receptors expressed on the cell membrane. In order to investigate the level of TRAIL-R1 (DR4, TNSFRSF10a) and TRAIL-R2 (DR5, TNSFRSF10b) expressed by HNSCC cell lines, three TRAIL sensitive cell lines (HN30, UMSCC74A and UMSCC11B) and three TRAIL resistant cell lines (HSC3, HSC3M3 and HN5) were stained with TRAIL receptor antibodies (HS101, anti-TRAIL-R1 and HS201, anti-TRAIL-R2) and analysed by flow cytometric analysis (FACS) to measure the level of expression of the receptor on the cells surface. The data was analysed using FlowJo program. These experiments were performed in collaboration with Dr Kerestin Papenfuss, Imperial College London.

The results shown in Figure 4.12 indicated that both TRAIL-R1 and TRAIL-R2 were expressed in all tested cell lines but at different levels. The level of expression is measured by the degree that receptor histogram shifts from isotype

histogram toward the right hand side. TRAIL-R2 was expressed at a higher level than TRAIL-R1 in all cell lines. TRAIL-R1 expression was higher in Smac59 sensitive (TRAIL resistant) cells, HSC3 and HSC3M3. These data indicated that sensitivity to TRAIL was independent of the level of its receptor expression. In conclusion, no clear association was observed between TRAIL death receptors expression and TRAIL sensitivity.

A



B

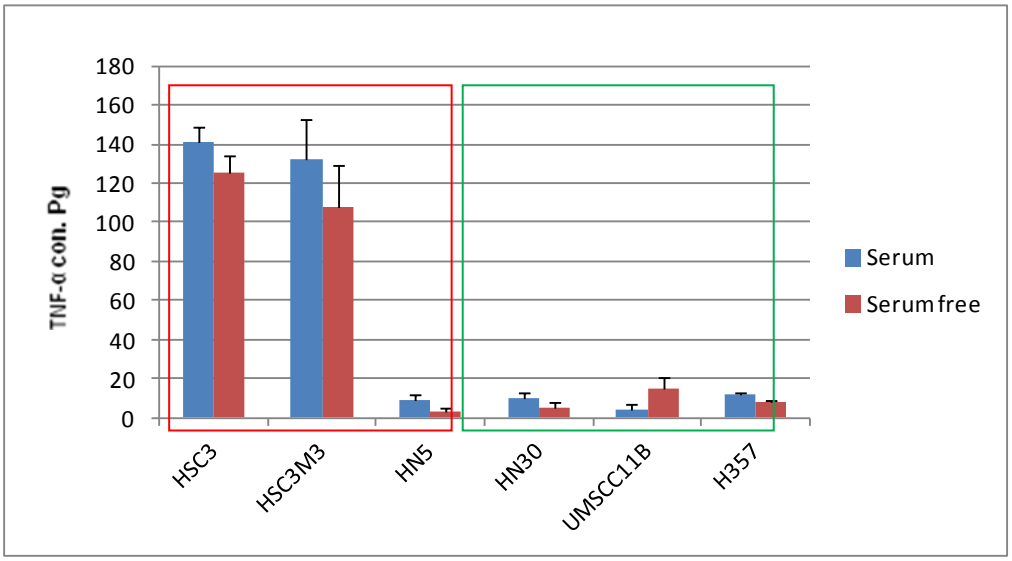


Figure 4.11 Level of secreted TNF-α in HNSCC cell lines

Three Smac59 cell lines (HSC3, HSC3M3 and HN5) and three TRAIL sensitive cell lines (HN30, H357 and UMSCC11B) were seeded in 12well plate and incubated at 37ºC and 5% CO_2 for 24 hours then the medium was replaced with fresh medium with or without serum. After 24 hours, the media were collected and were used to measure the level of secreted TNF-alpha using ELISA. A: standard curve created for the TNF-α used in ELISA. B: Graph showing the level of secreted TNF-α in the tested cell lines in the presence or absence of serum. All experiments were performed in duplicates. The results shown are the average of the TNF-α concentration in the tested samples. Red rectangle encloses the Smac59 sensitive (TRAIL resistant) cell lines and the green rectangle encloses the Smac59 resistant (TRAIL sensitive) cell lines.

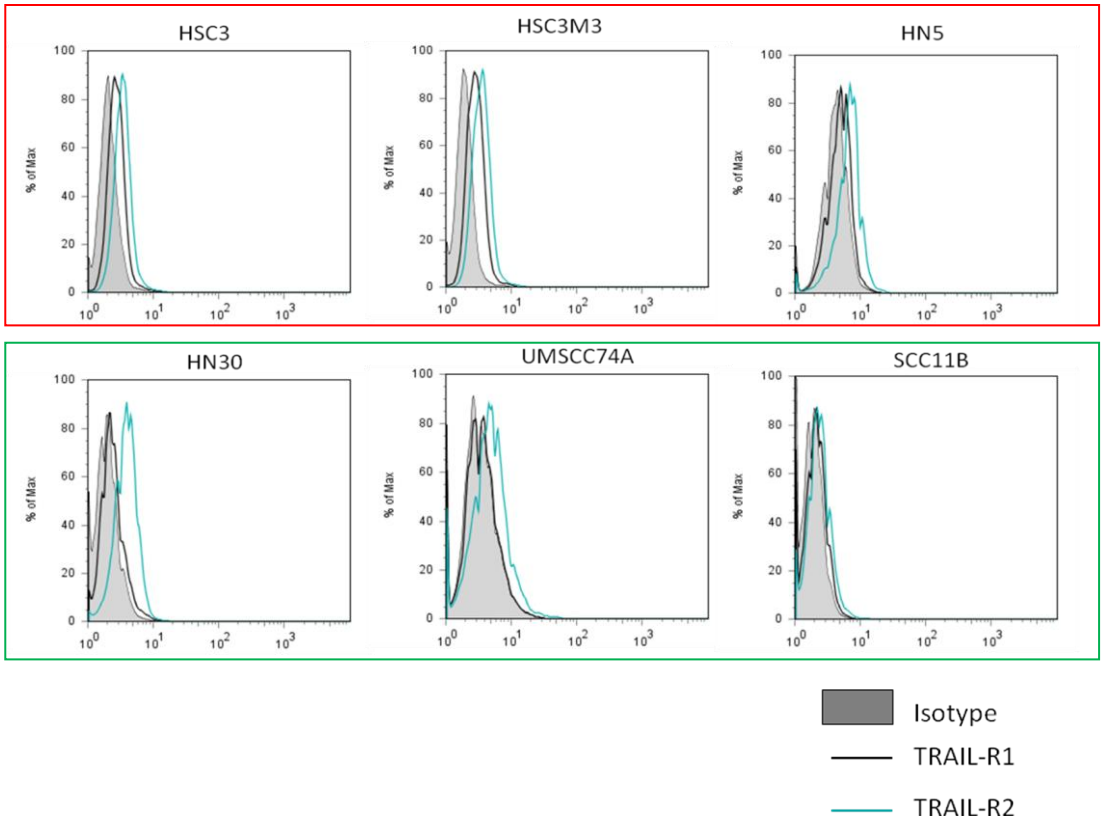


Figure 4.12 TRAIL receptors expression in HNSCC cell lines

Three Smac59 sensitive (TRAIL resistant) cell lines (HSC3, HSC3M3 and HN5) and three Smac59 resistant (TRAIL sensitive) cell lines (HN30, UMSCC74A and UMSCC11B) were seeded in T75 flasks until they were 80% confluent. Then the cells were stained with HS101, anti- TRAIL-R1 and HS201, anti-TRAIL-R2, and the receptor expression was analysed by FACS. Experiments were performed three different times and samples were tested in triplicate. The results shown are representative of the results obtained from the three experiments. Red rectangle encloses the Smac59 sensitive (TRAIL resistant) cell lines and the green rectangle encloses the Smac59 resistant (TRAIL sensitive) cell lines.

4.4 Gene and miRNA expression profiling

The results of the previous experiments suggested that a number of proteins that are involved in TRAIL and Smac59 apoptosis pathways might determine the sensitivity to TRAIL or Smac59 in HNSCC cell lines. Gene expression profiling analysis could increase the understanding of the molecular mechanisms involved in the sensitivity of HNSCC cell lines to TRAIL or Smac59 by testing the expression of large number genes which could provide an overall picture why HNSCC have this pattern of sensitivity to TRAIL and Smac59. The mRNA expression level of the tested proteins as well as other genes and miRNA targets that may affect response to TRAIL and Smac59 was investigated by microarray analysis.

Three Smac59 sensitive (TRAIL resistant) cell lines (HSC3, HSC3M3 and HN5) and five Smac59 resistant (TRAIL sensitive) cell lines (HN30, UMSCC74A, UMSCC74B, UMSCC11B and UMSCC22B) were seeded in 10cm dishes and incubated at 37ºC and 5%CO_2. At 24 and 48 hours after seeding, the cells were collected for RNA extraction. Gene expression profiling was performed using Illumina Human HT-12 v3 Expression BeadChip, which targets more than 25,000 annotated genes with more than 48,000 probes. The Chip provides genome-wide transcriptional coverage of well characterized genes, gene candidates and splice variants. Probes were designed using the RefSeq and UniGene data bases. miRNA expression profiling was performed using Illumina Human v2 miRNA Beadchip which could detect up to 97% (1,146) of known human miRNAs.

miRNA and mRNA microarray data for the samples listed in Table 4.1 were performed in Fondazione IRCCS Istituto Nazionale Tumori, Italy. The data were processed and analyzed by Katherine Lawler, PhD KCL/UCL Comprehensive Cancer Imaging Centre, King's College London. Six colorectal carcinoma cell lines available in the department were also included in the gene expression profiling on the same chip. The data for these samples presented in the figures as they were used for data normalization.

Figure 4.13 showed the hierarchical clustering of the normalized data using all probes and samples. mRNA data indicated that the HNSCC cell lines clustered separately from the colorectal carcinoma cell lines. Smac59 sensitive (TRAIL resistant) cell lines were clustered together. The Smac59 resistant (TRAIL sensitive) cell lines were clustered together except for the HN30 cell lines. This indicated that the clustered cell lines have similar mRNA expression profile. This confirmed that the expression profile and consequently the protein expression in different cell lines could be responsible for regulating their response to TRAIL and Smac59 treatment. The miRNA data showed different clustering pattern of tested cell lines in which UMSCC74A and UMSCC74B clustered separately from other cell lines. However, with the exception of HN30 cells, Smac59 sensitive (TRAIL resistant) cell lines were clustered together and Smac59 resistant (TRAIL sensitive) cell lines were clustered together.

Table 4.1 Data summary of the samples used for the gene expression profiling

Array ID*	Sample ID	TRAIL/ Smac mimetic status	Description
5050509009 _A	SCC74B (1)	TRAILsens Smacresist	HNSCC cell line ,24h
5050509009_ B	HN5 (1)	Smacsens TRAILresist	HNSCC cell line,24h
5050509009_C	HSC3M3 (1)	SMACsens TRAILresist	HNSCC cell line,24h
5050509009 _D	SCC22B (1)	TRAILsens Smacresist	HNSCC cell line,24h
5050509009_ E	HN5 (2)	Smacsens TRAILresist	HNSCC cell line, 48h
5050509009_ F	SCC74B (2)	TRAILsens Smacresist	HNSCC cell line, 48h
5050509009_ G	SCC74A (1)	TRAILsens SMACresist	HNSCC cell line,24h
5050509009_ H	HN30 (2)	TRAILsens SMACresist	HNSCC cell line, 48h
5050509009_ I	HN30 (1)	TRAILsens Smacresist	HNSCC cell line,24h
5050509009 _J	HSC3 (2)	Smacsens TRAILresist	HNSCC cell line, 48h
5050509009 _K	HSC3M3 (2)	Smacsens TRAILresist	HNSCC cell line, 48h
5050509009_ L	SCC11B (1)	TRAILsens Smacresist	HNSCC cell line,24h
5050509063_ A	SCC11B (2)	TRAILsens Smacresist	HNSCC cell line, 48h
5050509063_ B	SCC22B (2)	TRAILsens Smacresist	HNSCC cell line, 48h
5050509063 _C	HCT116 24h puma -/-	none	colorectal carcinoma
5050509063 _D	HCT116 24h p53 -/-	none	colorectal carcinoma
5050509063_ E	HCT116 3d h p53 wt	none	colorectal carcinoma
5050509063 _F	HCT116 24h h p53 wt	none	colorectal carcinoma
5050509063_ G	HSC3 (1)	Smacsens TRAILresist	HNSCC cell line,24h
5050509063_ H	HCT116 3d puma -/-	none	colorectal carcinoma
5050509063_I	HCT116 3d p53 -/-	none	colorectal carcinoma

*The array IDs have the format '[chipNumber]_[chipPosition (A-L)]

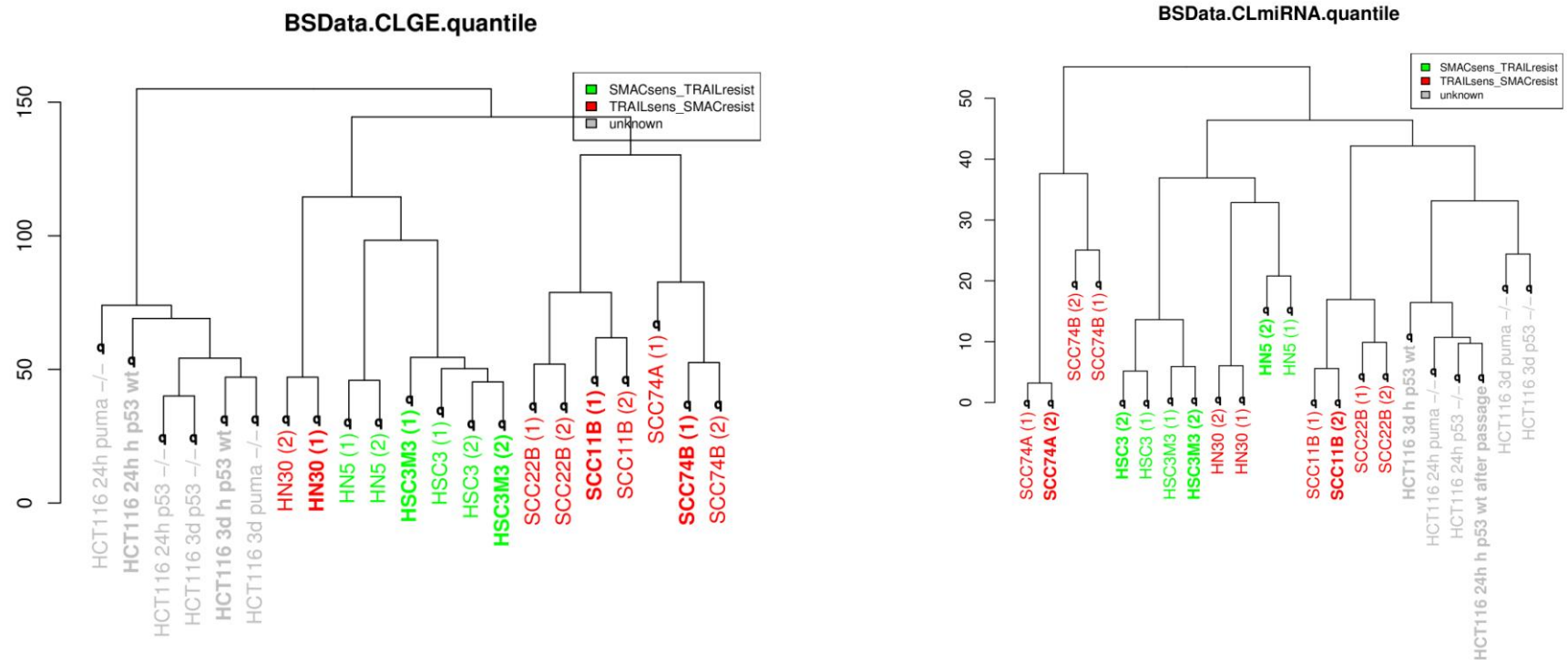


Figure 4.13 Unsupervised hierarchical clustering of all samples and all probes for gene and miRNA profiling results

Unsupervised hierarchical clustering for all tested samples on the same chip (HNSCC cell lines and colorectal cell lines). Green: Smac59 sensitive (TRAIL resistant) cell lines. Red: Smac59 resistant (TRAIL sensitive) cell lines. Grey: colorectal cell lines. Number 1 or 2 by cell line name indicates the 24 and 48 hours mRNA extraction after seeding samples respectively.

4.4.1 Gene expression profiling

Expression level of mRNA for proteins that are involved in TRAIL and Smac59 apoptosis pathways was analysed.

Figure 4.14 showed the unsupervised clustering and the gene expression of Smac/DIABLO, Mcl-1, CFLAR, TNFRSF10A (TR-1), XIAP, TNFRSF10C (DR-4), TNFRSF10D (DR-5), TRAF1, Bcl-2, caspase-8, TNF-α, caspase-3, TNFRSF10B (TR-2) and EGFR.

The data indicated that Smac/DIABLO was highly expressed in Smac59 sensitive (TRAIL resistant) cells than Smac59 resistant (TRAIL sensitive) cell lines. With the exception of HN30 cell line, Mcl-1 was expressed at higher levels in Smac59 sensitive (TRAIL resistant) cell lines. With the exception of HN30 cell line, CFLAR (cFLIP) was higher in Smac59 resistant (TRAIL sensitive) cell lines. With the exception of HN5 cells, TNF and TNFR2 were expressed at higher levels in Smac59 sensitive (TRAIL resistant) cells while TRAF1 expression pattern was not associated with Smac59 sensitivity. With the exception of HN30 cell line, TNFRSF10A (TR-1) and TNFRSF10B (TR-2) showed higher levels of expression in Smac59 sensitive (TRAIL resistant) cells. No clear association between TNFRSF10C (DR-4) and TNFRSF10D (DR-5) and TRAIL or Smac59 sensitivity was observed.

XIAP was highly expressed in Smac59 sensitive (TRAIL resistant) cells. With the exception of HN5 and HN30 cell lines, Bcl-2 was overexpressed in Smac59 resistant (TRAIL sensitive) cell lines. With the exception of HN5 and HN30 cell lines, caspase-8 was highly expressed in Smac59 resistant (TRAIL sensitive) cell lines while caspase-3 expression was not correlated to TRAIL or Smac59 sensitivity. With the exception of HN30 cell line, EGFR was highly expressed in Smac59 sensitive (TRAIL resistant) cell lines.

In summary, the results from gene expression profiling indicated that, Smac/DIABLO, Mcl-1, TRAF-2, TNF, TR-1, TR-2, XIAP and EGFR overexpression were associated with Smac59 sensitivity and Bcl-2, CFLIP and

caspase-8 overexpression were associated with TRAIL sensitivity. Results of TNF-α, Bcl-2, c-FLIP, Bcl-2, caspase-8 and EGFR confirmed the results obtained from the protein analysis. On the other hand, the difference in the expression of TNFRSF10a (TR-1), TNFRSF10b (TR-2) and XIAP was not clearly identified by the protein analysis. Taken together, this data suggested that the expression profile of different proteins in HNSCC cell lines could be responsible for regulating their response to TRAIL and Smac59. However, the exact role of each of these proteins in this process required further investigations.

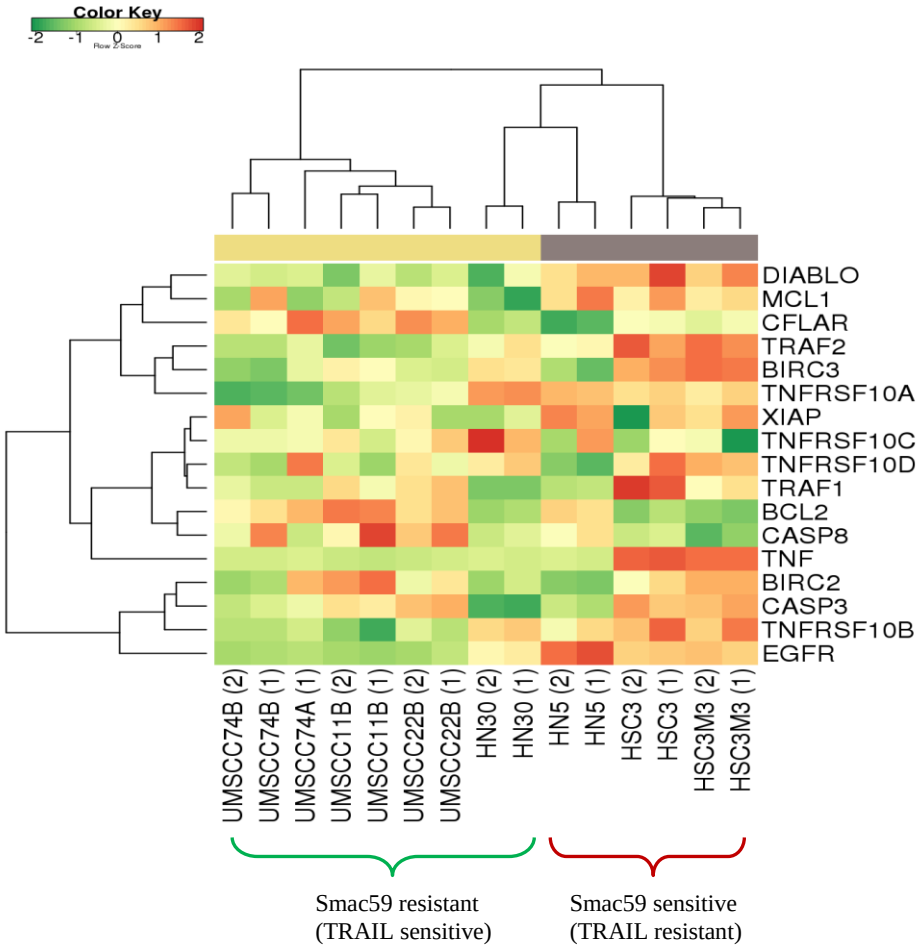


Figure 4.14 Unsupervised hierarchical clustering

Unsupervised hierarchical clustering of selected TRAIL/Smac apoptosis pathway members in selected cell lines (HNSCC cell lines). Horizontal colour bar indicates TRAIL/Smac sensitivity; yellow=TRAIL sensitive/Smac59 resistant; grey=Smac59 sensitive/TRAIL resistant. The orange and green colours represent the scale of expression of the tested genes. Number 1 or 2 by cell line name indicates the 24 and 48 hour mRNA sampling of the cells respectively.

4.4.2 miRNA expression profiling

Differential miRNAs expression in Smac59 sensitive (TRAIL resistant) cell lines versus Smac59 resistant (TRAIL sensitive) cell lines was analysed to identify potential biomarkers for predicting sensitivity to TRAIL or Smac59.

The data shown in Figure 4.15 illustrating the unsupervised clustering and the miRNA expression in TRAIL sensitive versus Smac59 sensitive cell lines, showed 15 miRNA that were upregulated (A) and 7 miRNA which were downregulated (B) in Smac59 resistant (TRAIL sensitive) cell lines compared with Smac59 sensitive (TRAIL resistant) cell lines (Table 4.2). This data confirmed that the sensitivity/resistant pattern of HNSCC cell lines to TRAIL and Smac59 appears to be explained by the differential expression of a number of miRNA. These miRNA could be ideal candidates as biomarkers for this sensitivity and will need further validation that is not covered in this thesis.

Table 4.2 Selected miRNA significantly up or down regulated in HNSCC cell lines

Upregulated miRNA in Smac59 resistant (TRAIL sensitive) cell lines (15)	Downregulated miRNA in Smac59 resistant (TRAIL sensitive) cell lines (7)
Hsa-mir-9* Hsa-mir-9 Hsa-mir-212 Hsa-mir-326 Hsa-mir-99a Solexa-6676-127 Hsa-mir-10a* Hsa-mir-328 Hsa-mir-195 Hsa-mir-126* Hsa-mir-100 Hsa-mir-10a Solexa-3126-285 Hsa-mir-653:9.1 Hsa-mir-489	Hsa-mir—21* Hsa-mir-189:9.1 Hsa-mir-1301 Hsa-mir-455-5p Hsa-mir-33b Hsa-mir-124 Hsa-mir-486-3p

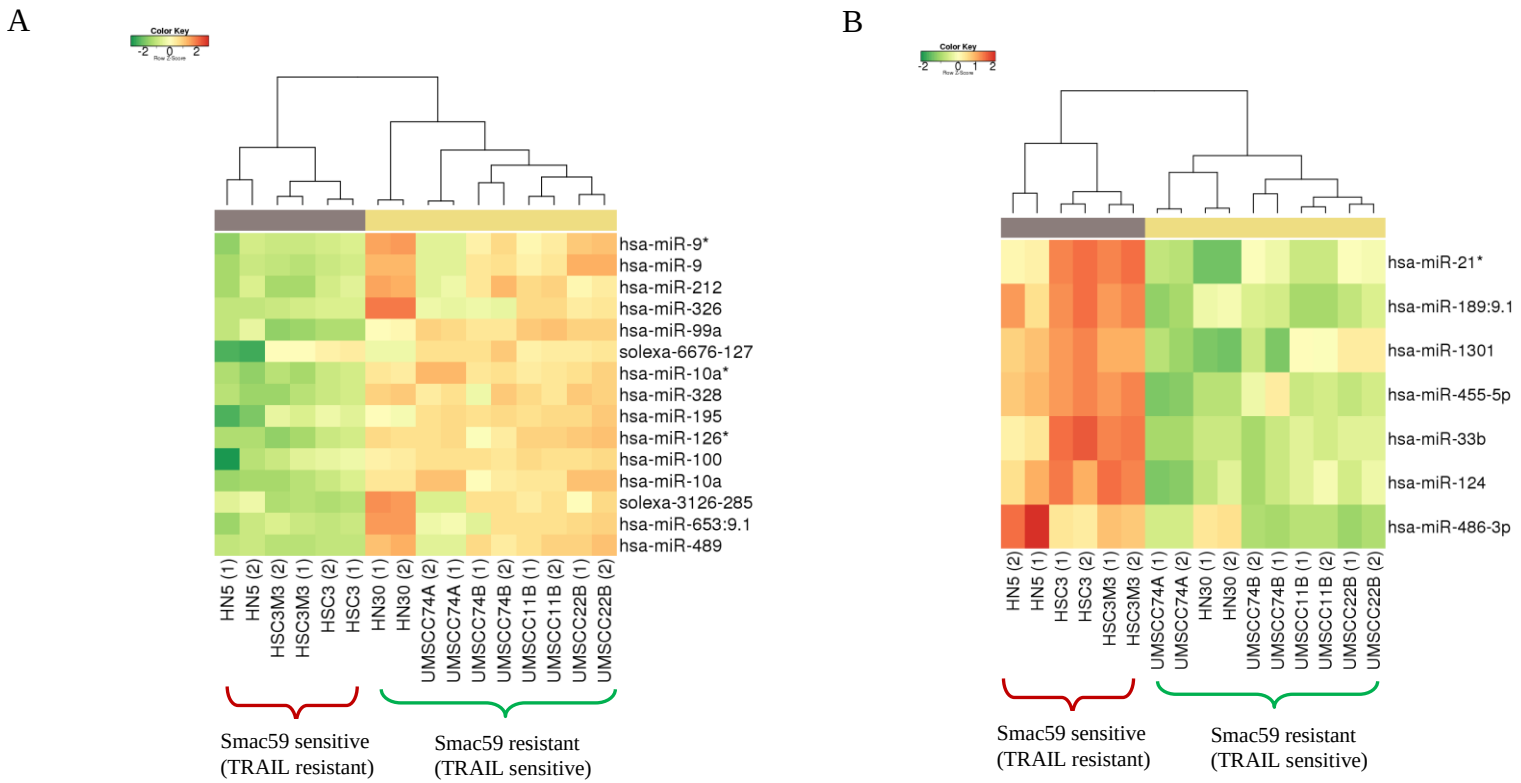


Figure 4.15 Selected miRNA significantly up or down regulated in HNSCC cell lines

A: upregulated miRNAs, B: downregulated miRNAs in Smac59 sensitive (TRAIL resistant) versus Smac59 resistant (TRAIL sensitive) cell lines. Heatmaps are displayed after hierarchical clustering of samples for the selected miRNAs. Horizontal colour bar indicates TRAIL/Smac sensitivity; yellow= Smac59 resistant (TRAIL sensitive); grey=Smac59 sensitive (TRAIL resistant). The orange and green colours represent the scale of expression of the tested miRNA; green is low and red is high as scale bar shows. Number 1 or 2 by cell line name indicates the 24 and 48 hours mRNA sampling of the cells respectively.

In order to investigate whether the differential expression of miRNAs mentioned above is affecting the gene expression in the tested cell lines, two miRNAs from each group, hsa-mir-9* and hsa-mir-9 from upregulated group and hsa-mir-21* and hsa-mir-124 from the downregulated group, were selected for further statistical analysis using the LIMMA package (R/Bioconductor). The validated targets for each miRNA were obtained from a microRNA database on validated and predicted miRNA targets website called miRWALK. These validated targets were subjected to statistical analysis to identify genes which are significantly differentially expressed between the treatment-response groups. The subset of miRNA targets which were detected as significantly differentially expressed between the treatment-response groups are shown in Table 4.3. From the list of genes that previously identified as differentially expressed between Smac59 sensitive/TRAIL resistant and Smac59 resistant/TRAIL sensitive cells (Figure 4.14), EGFR is the only gene presented as differentially expressed between the two treatment groups. Further investigation is needed to validate these identified genes and to investigate their role in determining the sensitivity/resistant to Smac59 and TRAIL in HNSCC cell lines.

Table 4.3 List of miRNA target genes differentially expressed in TRAIL sensitive/Smac resistant cells

miRNA	Genes upregulated in TRAIL sensitive/Smac resistant cells	Genes downregulated in TRAIL sensitive/Smac resistant cell
Hsa-mir-9*	MEIS2 CDKN2D	BANF1 DNMT1
Hsa-mir-9	MEIS2 CDKN2D NOG	DICER1 DNMT1
Hsa-mir-21*	SRGN BMPR2 ARIH2	LAMC2 **EGFR** DICER1 SIP1 PDPN PRMT5 DGCR8 HOXD10 DICER2 NRAS PTEN DNMT1 TM9SF3
Hsa-mir-124	PODXL C11or F75 SEC11 H3F3B ACPL2 TRIM2 CDKN2D PFTK1 ACAA2 MDFIC	EFNB1 LRRC8 CAV1 DICER1 GCLC TMEM14A PEC1 C12 orf49 DGCR8 BANF1 HIST1H3G FBLN2 GPSM2 PRMS1

In conclusion, identification of biomarkers for predicting the response of HNSCC cell lines to TRAIL and Smac59 was investigated. This was performed by associating the expression of several proteins, genes and miRNA in HNSCC cell lines using several different experimental techniques. The data obtained suggested:

- TNF-α, MCl-1, XIAP, Smac/DIABLO, TR-1, TR-2 and EGFR were overexpressed in Smac59 sensitive (TRAIL sensitive) cell lines and underexpressed in Smac59 resistant (TRAIL sensitive) cell lines.
- Bcl-2, Caspase-8 and cFLIP were overexpressed in Smac59 resistant (TRAIL sensitive) and underexpressed in Smac59 sensitive (TRAIL sensitive) cell lines.
- 15 miRNA were significantly upregulated and 7 were significantly downregulated in the Smac59 resistant (TRAIL sensitive) versus Smac59 sensitive (TRAIL sensitive) cell lines.
- These proteins and miRNAs could be potential biomarkers for TRAIL or Smac59 sensitivity in HNSCC cell lines and possibly other cells.

In the next chapter, validation and functional analysis for number of these proteins will be addressed in order to investigate the mechanisms underlying TRAIL and Smac59 induced apoptosis.

5 Results III

Mechanisms of TRAIL and Smac mimetic-induced apoptosis in HNSCC cell lines

In the previous chapter, a number of proteins were found to be involved in determining the TRAIL/Smac59 sensitivity/resistance pattern in the tested HNSCC cell lines. TNF-α, Bcl-2 and XIAP were selected for further functional studies. HSC3M3 was selected as a candidate Smac59 sensitive cell line and UMSCC11B as a candidate TRAIL sensitive cell line to investigate the role of TNF-α, Bcl-2 and XIAP proteins in TRAIL and Smac59 induced apoptosis.

TRAIL induces apoptosis mainly via activation of the extrinsic pathway, which is initiated by binding of TRAIL to TRAIL-R1 and R2 receptors leading to activation of caspase-8 and consequently caspase-3 activation. In some cell lines extrinsic pathway activation is not sufficient to accomplish apoptosis; in these cells the intrinsic pathway will also be activated. Smac mimetics were originally designed to mimic the interaction between XIAP and its antagonist Smac/DIABLO. However, the exact mechanism by which Smac mimetics induce apoptosis is still not fully understood.

5.1 Role of TNF-α in TRAIL/ Smac59 induced apoptosis

It has been shown that Smac mimetics kill cancer cells via activation of the NF-κB pathway which leads to the production of TNF-α, the newly synthesized TNF-α is subsequently secreted to activate TNF receptors through autocrine or paracrine pathways (LaCasse et al., 2008).

Our results indicated that the level of secreted TNF-α in HNSCC cell lines correlated with their sensitivity to Smac59. In order to understand the role of TNF-α in TRAIL and Smac59 induced apoptosis, the effect of TRAIL and Smac59 treatment on the level of secreted TNF-α was investigated together with the effect of adding recombinant TNF-α or inhibition of secreted TNF-α.

5.1.1 Effect of TRAIL and Smac59 treatment on TNF-α secretion in HNSCC cell lines

Three Smac59 sensitive (TRAIL resistant) cell lines (HSC3, HSC3M3 and HN5) and three Smac59 resistant (TRAIL sensitive) cell lines (HN30, H357 and UMSCC11B) were tested for the level of secreted TNF-α before and after treatment with TRAIL and Smac59. Equal numbers of cells were seeded in 12well plates in 1ml media, incubated at 37ºC and 5% CO_2 for 24hours. Then the medium was replaced with fresh medium containing TRAIL 50ng/ml or Smac59 50nM. The cells were then incubated for 24hours and the medium was collected and stored at -70ºC. Level of TNF-α in the medium was measured by ELISA as described in material and methods.

The results shown in Figure 5.1 indicated that in Smac59 sensitive (TRAIL resistant) cell lines (HSC3, HSC3M3 and HN5), Smac59 treatment induced autocrine TNF-α secretion up to six folds over the endogenous level. In Smac59 resistant (TRAIL sensitive) cell lines (HN30, H357 and UMSCC11B), Smac59 induced TNF-α secretion at a much lower level. TRAIL treatment had no significant stimulatory effect on the TNF-α secretion in all tested cell lines. These results suggested that autocrine secretion of TNF-α may be one of the mechanisms by which Smac59 induce apoptosis in HNSCC cell lines. Whether lack of TNF-α in Smac59 resistant (TRAIL sensitive) cell lines is responsible for their resistance required a separate investigations.

5.1.2 Effect of adding recombinant TNF-α in HNSCC cell lines

The results in section 5.1.1 opened two lines of investigation. The first was to investigate whether TNF-α could replace Smac59 in the sensitive cell lines to induce cell death. Recombinant human TNF-α was added to HSC3M3 and UMSCC11B cell lines. Cells were seeded in 96well plates, incubated at 37ºC and 5% CO_2 for 24hours, then the medium was removed and was replaced with fresh medium containing recombinant h-TNF-α (10ng/ml), TRAIL (50ng/ml) or Smac59

(50nM). Cells were incubated for 24 hours then cell viability was tested using MTT assay.

The results in Figure 5.2A showed that TNF-α inhibited HSC3M3 cell viability by about 20% while Sma59 induced approximately 70% loss of cell viability. TNF-α had a minimal cytotoxic effect on UMSCC11B (Smac59 resistant/TRAIL sensitive cell line). This data suggested that Smac59-induced TNF-α autocrine secretion in HNSCC is not the only mechanisms by which Smac59 induces apoptosis in these cell lines.

The second line of investigation was to examine whether low Smac59 induced TNF-α secretion in Smac59 resistant (TRAIL sensitive) cell lines is the cause of their resistance to Smac59 treatment. Smac59 resistant (TRAIL sensitive) cell line, UMSCC11B, was seeded in 96well plate, incubated at 37ºC and 5% CO_2 for 24hours. Then the medium was removed and replaced with fresh medium containing recombinant h-TNF-α at (10, 5, 2.5, 1.25, 0.625, 0.3125, 0.15625 ng/ml) concentrations alone or in combination with TRAIL (50ng/ml) or Smac59 (50nM). After 24 hours the cell viability was measured using the MTT assay.

The results shown in Figure 5.2B indicated that adding rh-TNF-α to Smac59 reduced cell viability of UMSCC11B (Smac59 resistant/TRAIL sensitive) by 70-80%. This effect was even greater than the effect induced by TRAIL treatment alone or combined with rh-TNF-α. As UMSCC11B is resistant to Smac59 and as shown in Figure 5.1, it secretes a low level of TNF-α after Smac59 treatment, this result suggested that TNF-α sensitises resistant cells to Smac59 and further confirmed that TNF-α is important for Smac59 induced apoptosis in HNSCC cell lines.

5.1.3 Effect of antagonizing TNF-α in HNSCC cell lines

To further confirm the importance of TNF-α in Smac59 sensitivity in HNSCC cell lines, the effect of TNF-α inhibition on Smac59 sensitivity in HNSCC cell lines was investigated. The inhibition of TNF-α was performed either by using

neutralizing antibody to inhibit its interaction with its receptors, or by using blocking antibodies to TNF-α receptors.

HSC3M3 cell line (Smac59 sensitive) was seeded in 96 well plate, incubated at 37ºC and 5%CO_2 for 24hours. Then the medium was replaced with fresh medium containing either neutralizing antibody (anti-h-TNF-α, 10ug/ml, InvivoGen) or blocking antibody (anti-TNFR1, 20 ug/ml, ABD Serotec). The cells were incubated with the antibody for 1hour before adding Smac59 (50nM). After 24 hours cell viability was measured using MTT assay.

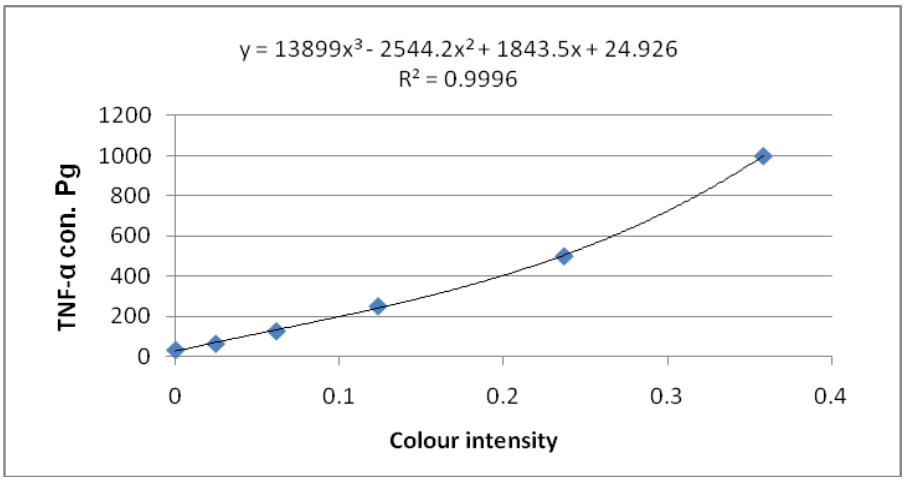
The results illustrated in Figure 5.3A indicated that the neutralizing antibody was not able to protect the HSC3M3 cells from the cytotoxic effect of Smac59. Smac59 inhibited cell growth by 60% in the presence or absence of TNF-α neutralizing antibody. Similarly, as shown in Figure 5.3B, the anti-TNFR1 blocking antibody was unable to inhibit Smac59 induced apoptosis in the HSC3M3 cell line.

In MCF-7 breast cancer cell lines, TNF-α activates NF-κB pathway through ubiquitin-mediated degradation of IκBs (Machuca et al., 2006). This effect should be inhibited when TNF-α activity is blocked by inhibitory antibodies. In order to test whether TNF-α and its inhibitory antibodies were functional, MCF-7 cells were seeded in 12well plates and were incubated at 37ºC and 5%CO_2 for 24hours. The medium was removed and replaced with fresh medium containing neutralizing or blocking antibody. The cells were incubated for one hour before adding recombinant h-TNF-α. After 30min, cell lysates were collected and tested for IκB-α expression using western blot analysis. The data shown in Figure 5.3C indicated that TNF-α induced down regulation of IκB-α in MCF-7. This downregulation was inhibited when the cells were pre-treated with the inhibitory antibodies. This confirmed the activity of rh-TNF-α and the inhibitory antibodies and supported our observations.

In conclusion, the results obtained suggested that TNF-α could be a marker of Smac59 sensitivity and that TNF-α could be used to sensitise Smac59 resistant cells to Smac59 induced apoptosis. However, the exact mechanism by which Smac59 utilizes the TNF-α pathway is not clearly understood. TNF-α alone was

unable to induce cell death as seen with Smac59. Furthermore, blocking TNF-α did not inhibit Smac59-induced apoptosis. Collectively, these results suggested that TNF-α was important for Smac59-induced apoptosis. The exact mechanism by which TNF-α is involved in Smac59-induced apoptosis needs further investigation.

A



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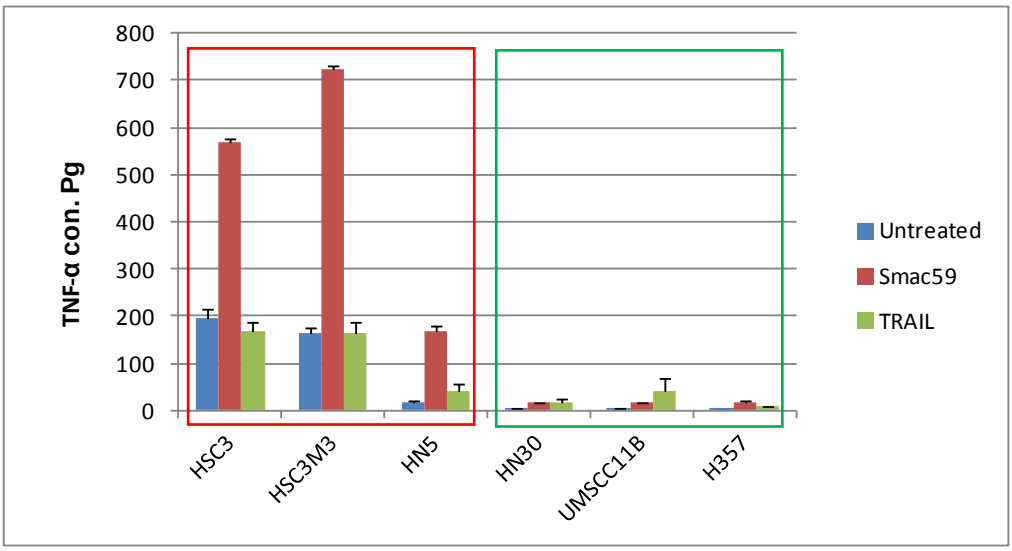


Figure 5.1 Effect of TRAIL and Smac59 treatment on TNF-α secretion in HNSCC cell lines

Three Smac59 sensitive cell lines and three TRAIL sensitive cell lines were seeded in 12well plates and incubated at 37ºC and 5%CO_2 for 24hours then the medium was replaced with fresh medium containing TRAIL (50ng/ml) or Smac59 (50nM). After 24 hours, the media were collected and were used to measure the secreted TNF-α using ELISA. A: standard curve created for the TNF-α standard used in ELISA. B: The level of secreted TNF-α in the tested cell lines. All experiments were performed in duplicate. The results shown are the average of the TNF-α concentration in the tested samples. Red rectangle encloses the Smac59 sensitive (TRAIL resistant) cell lines and the green rectangle encloses the Smac59 resistant (TRAIL sensitive) cell lines.

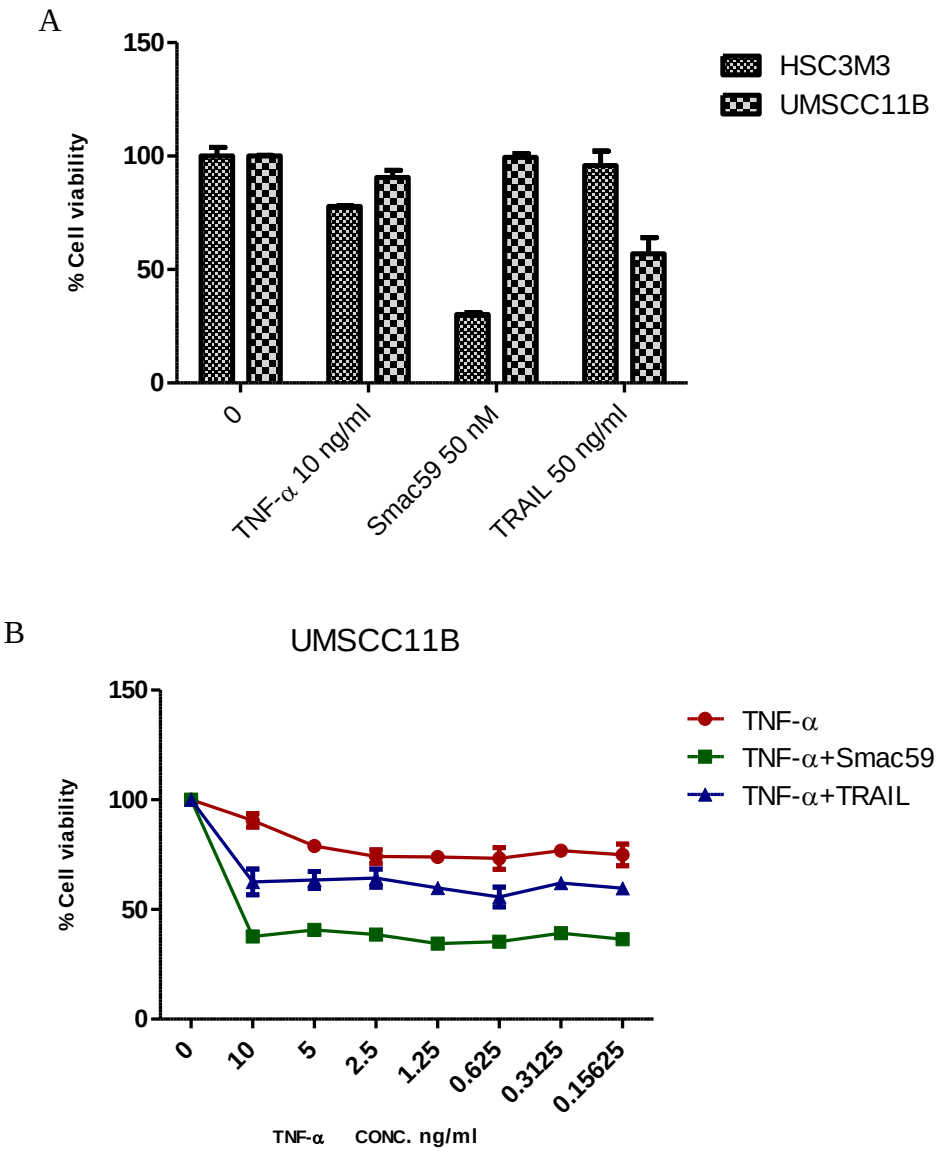


Figure 5.2 Effect of TNF-α addition on the viability of HNSCC cell lines

A: HSC3M3 and UMSCC11B cells were seeded in 96 well plates and incubated at 37ºC and 5% CO_2 for 24hours then the medium was replaced with fresh medium containing rh-TNF-α, (10ng/ml), TRAIL (50ng/ml) or Smac59 (50nM). After 24hours the cell viability was measured using MTT assay. B: UMSCC11B cells were seeded in 96 well plate and incubated at 37ºC and 5% CO_2 for 24hours then the medium was replaced with fresh medium containing rh-TNF-α (10, 5, 2.5, 1.25, 0.625, 0.3125, 0.15625 ng/ml) alone or with TRAIL 50ng/ml or Smac59 50nM. After 24 hours the cell viability was measured using MTT assay. The results are shown as the percentage of the viable cells after treatment with respect to viable untreated cells. All experiments were performed in triplicate, error bars indicate SD.

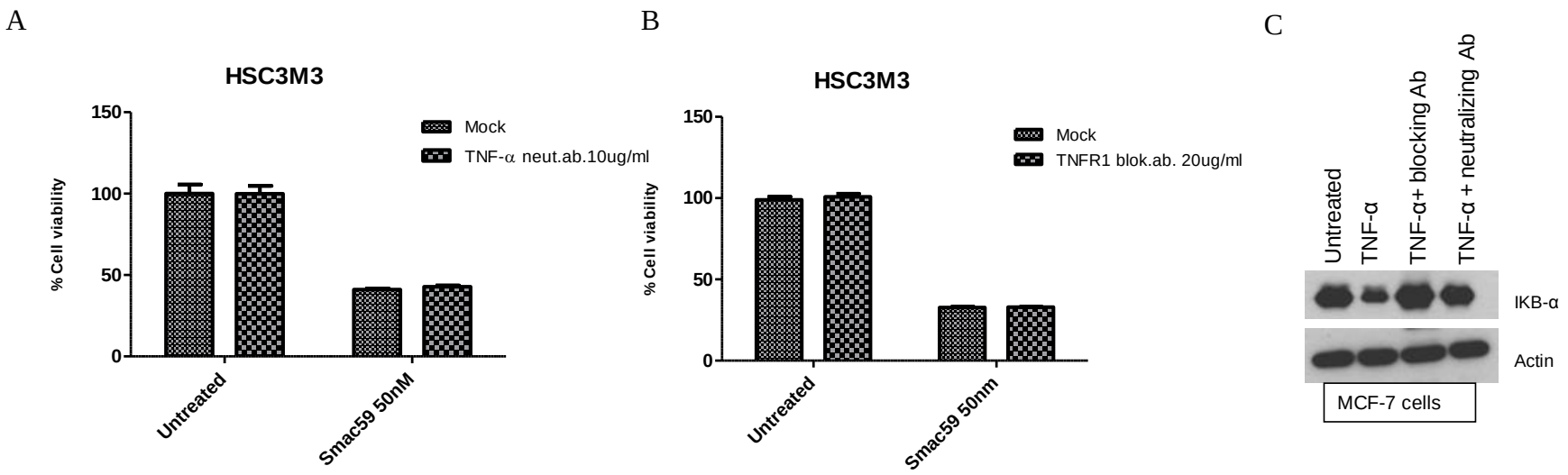


Figure 5.3 Effect of TNF-α inhibition on the HNSCC cell lines

A,B: HSC3M3 cells were seeded in 96well plates and incubated at 37ºC and 5%CO_2 for 24hours then the medium was replaced with fresh medium containing either neutralizing antibody (anti-h-TNF-α, 10ug/ml, InvivoGen) or blocking antibody (anti-TNF-R1, 20ug/ml, ABD Serotec). The cells were incubated with the antibody for 1hour before adding Smac59 (50nM). After 24 hours cell viability was measured using MTT assay. C: MCF-7 cells were seeded in 12well plates and were incubated at 37ºC and 5%CO_2 for 24hours. Then the medium was removed and replaced with fresh medium containing neutralizing or blocking antibody. The cells were incubated for one hour before adding recombinant h-TNF-α. After 30min, cell lysates were collected and tested for IκB-α expression using western blotting analysis. The results are shown as the percentage of the viable cells after treatment with respect to viable untreated cells. All experiments were performed in triplicate, error bars indicate SD.

5.2 Role of Bcl-2 in TRAIL/Smac59 induced apoptosis

Bcl-2 is an anti-apoptotic member of Bcl-2 family; it prevents membrane disruption of the mitochondria as part of intrinsic apoptosis pathway. Overexpression of Bcl-2 molecules has been shown to cause TRAIL resistance (Fulda et al., 2002). As shown in figure 5.5, Bcl-2 was overexpressed in four of our Smac59 resistant (TRAIL sensitive) HNSCC cell lines, while Smac59 sensitive (TRAIL resistant) cell lines had undetectable levels of Bcl-2 protein. The role of Bcl-2 up or downegulation in HNSCC cell lines and its connection to sensitivity/resistant to TRAIL and Smac59 were further investigated.

5.2.1 Effect of Bcl-2 overexpression on Smac59 sensitivity in Smac59 sensitive cell lines

To investigate the role of Bcl-2 overexpression on Smac59 sensitivity in HNSCC cell lines, stable bcl-2 expressing mixed populations from the HSC3M3 cell line were established for the purpose of this study (Figure 5.4C). HSC3M3 is a Smac59 sensitive cell line and it showed undetectable levels of endogenous Bcl-2. HSC3M3 and HSC3M3-Bcl-2 cells were seeded in 96well plates and incubated at 37ºC and 5%CO_2 for 24hours then the medium was replaced with fresh medium containing Smac59 at (2, 1, 0.5, 0.25, 0.125nM) concentrations. The cells were incubated for 24hours then the cell viability was tested with MTT assay.

As illustrated in Figure 5.4A and Figure 5.4B, Bcl-2 overexpression in HSC3M3 cells did not protect the cells from Smac59-induced apoptosis. The cytotoxic effect of Smac59 was similar in both HSC3M3 and HSC3M3-Bcl-2 cells suggesting that bcl-2 may not be involved in the regulation of Smac59-induced apoptosis and that Smac59 may induce cell death by acting downstream or independently of Bcl-2.

5.2.2 Effect of Bcl-2 overexpression on TRAIL sensitivity in TRAIL sensitive cell lines

Four (UMSCC74A, UMSCC74B, UMSCC11B and UMSCC22B) out of six Smac resistant (TRAIL sensitive) HNSCC cell lines expressed high levels of Bcl-2 (Figure 4.5) suggesting a possible role for Bcl-2 in TRAIL-induced apoptosis. While two (HN30, H357) had undetectable Bcl-2 expression. In order to investigate the role of Bcl-2 overexpression on TRAIL sensitivity in HNSCC cell lines, stable Bcl-2 expressing mixed populations from the H357 cell line were established in the department by Dr Marcella Flinterman (Figure 5.5D). H357 and H357-Bcl-2 cells were seeded in 96 well plates and incubated at 37ºC and 5%CO_2 for 24hours then the medium was replaced with fresh medium containing TRAIL at (100, 50, 25, 12.5, 6.25, 3.125, 1,65ng/ml) concentrations. The cells were incubated for 24hours then the cell viability tested with MTT assay.

The results shown in Figure 5.5A and Figure 5.5B indicated that TRAIL induced 25% cell death in H357-Bcl-2 cell compared with 75% cell death induced in H357 cells. This difference in the level of cytotoxicity was statistically significant (p value <0.001) (Figure 5.5C). These findings suggested that Bcl-2 overexpression inhibited TRAIL-induced apoptosis in H357-Bcl-2 cells indicating that TRAIL-induced apoptosis in this cell lines was regulated by Bcl-2. H357 cells had a lower caspase-8 level than other Smac59 resistant (TRAIL sensitive) cell lines (Figure 4.7), which may indicate that TRAIL-induced cell death in H357 cell lines may be mediated through activation of both extrinsic and intrinsic pathways and that H357 cells are type II cells.

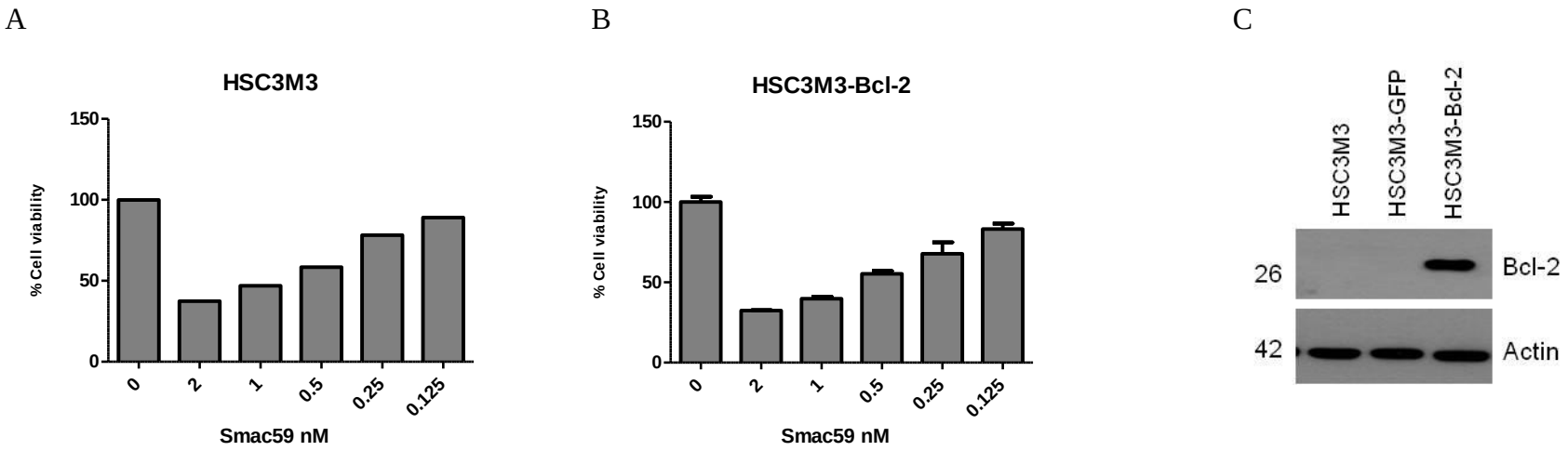


Figure 5.4 Effect of Bcl-2 overexpression in Smac59 sensitive cells

A,B: HSC3M3 and HSC3M3-Bcl-2 cells were seeded in 96 well plates and incubated at 37ºC and 5%CO_2 for 24hours then the medium was replaced with fresh medium containing Smac59 at (2, 1, 0.5, 0.25, 0.125nM) concentrations. The cells were incubated at 37ºC and 5% CO_2 for 24hours then the cell viability tested with MTT assay. C: Western blotting showing Bcl-2 expression in the HSC3M3 and HSC3M3-Bcl-2 cells. The results are shown as the percentage of the viable cells after treatment with respect to viable untreated cells. All experiments were performed in triplicate, error bars indicate SD.

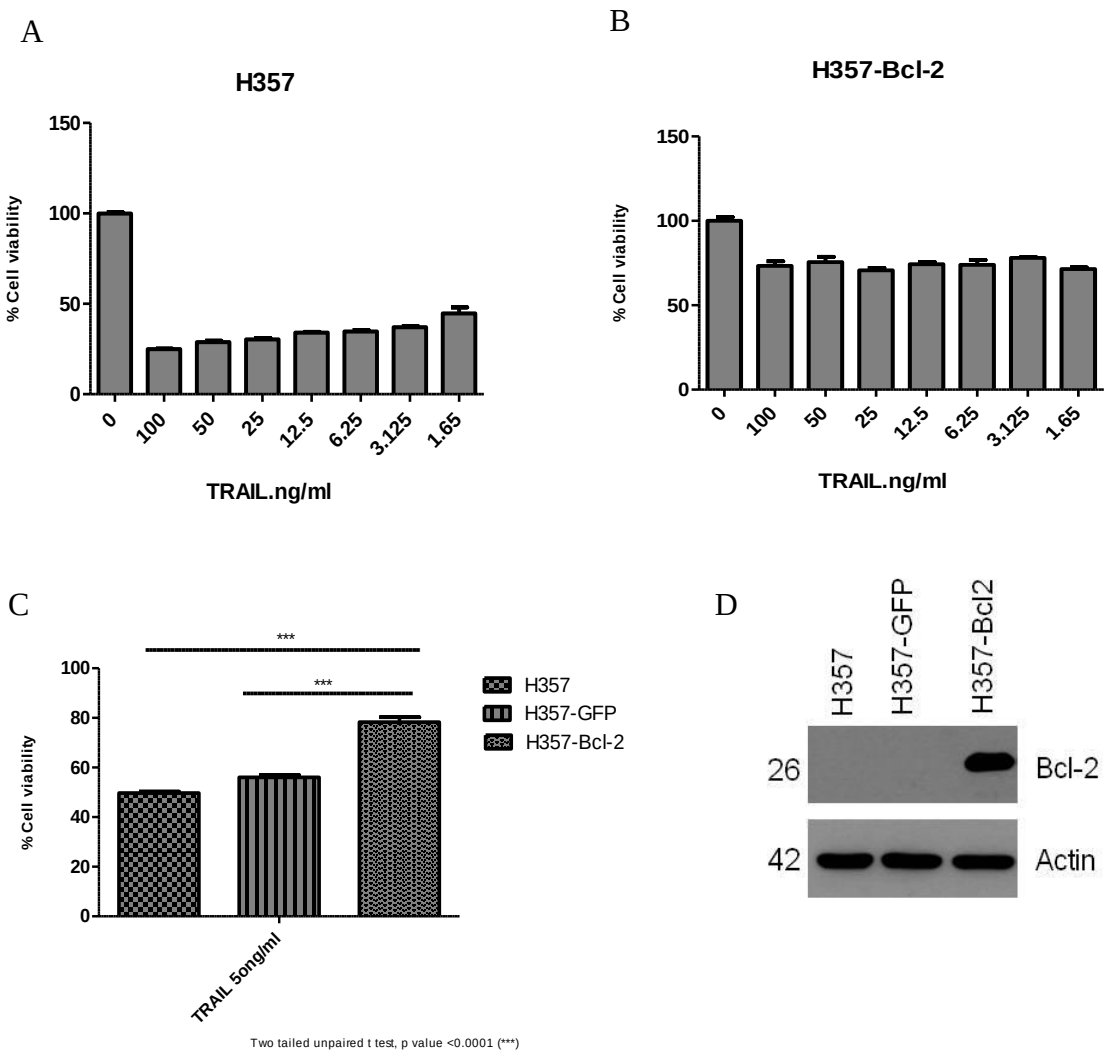


Figure 5.5 Effect of Bcl-2 overexpression in TRAIL sensitive cell lines

A,B: H357 and H357-Bcl-2 cells were seeded in 96well plates and incubated at 37ºC and 5%CO_2 for 24hours then the medium was replaced with fresh medium containing TRAIL at (100, 50, 25, 12.5, 6.25, 3.125, 1.65ng/ml) concentration. The cells were incubated for 24hours then the cell viability tested with MTT assay. C: H357, H357-GFP and H357-Bcl-2 cells were treated with TRAIL (50ng/ml). Cell viability tested with MTT assay. D: Western blotting showing Bcl-2 expression in the H357 and H357-Bcl-2 cells. The results are shown as the percentage of the viable cells after treatment with respect to viable untreated cells. All experiments were performed in triplicate, error bars indicate SD. * two tail unpaired t-test *p* value < 0.0001.

5.3 Role of IAPs in the TRAIL/Smac59 induced apoptosis

IAPs are efficient suppressors of apoptosis induced by variety of stimuli, including death receptor activation, growth factor withdrawal, ionizing radiation, viral infection and genotoxic damage (Hunter et al., 2007). Since IAPs function at the convergence of both the mitochondrial pathway and death receptor pathway, they are described as an apoptosis brake and IAP antagonists function to release the brake (Dai et al., 2009a). XIAP is the first well characterized IAP family member due to its potent anti-apoptosis activity. The importance of IAPs in the regulation of apoptosis induction by TRAIL and Smac59 in HNSCC cell lines was investigated.

5.3.1 TRAIL and Smac59 induce Caspase dependant cell death

In order to investigate the mechanism by which TRAIL and Smac59 induce cell death in HNSCC cells lines, HSC3M3 and UMSCC11B cells were seeded in 12well plates, incubated for 24hours at 37ºC and 5%CO_2. Then medium was replaced by a fresh medium containing TRAIL (50ng/ml) or Smac59 (50nM). After 2, 4, 8 and 12hours of incubation, the cells were lysed and subjected to western blot analysis to investigate capase-3 activation and apoptosis induction as marked by caspase-3 mediated cleavage of Poly (ADP-ribose) polymerase (PARP). PARP is a family of several proteins involved in a number of cellular process mainly DNA repair and apoptosis. Activated caspase-3 induces PARP cleavage which results in its inactivation. Detection of cleaved fragments of PARP (89 kDa) by western blot analysis indicates that the cells were undergoing apoptosis.

The results shown in Figure 5.6 indicated that TRAIL induced caspase-3 activation in TRAIL sensitive cell line UMSCC11B. Caspase-3 activation was observed at four hours after adding TRAIL to the cells; this activation reached the maximum level at 12hours. Cleavage of PARP was observed concurrently with caspase-3 activation at 4 hours. Similarly, Smac59 induced caspase-3 activation in HSC3M3 Smac59 cell line. However caspase-3, activation and PARP cleavage was observed after 8hours. This data suggested that TRAIL and Smac59 induced caspase-dependant apoptosis in HNSCC cell lines.

5.3.2 Effect of TRAIL and Smac59 on IAPs expression in HNSCC cell lines

IAPs overexpression is a main cause of cancer cells evading apoptosis. In order to induce apoptosis in these cells, IAPs activity could be inhibited either by endogenous Smac/DIABLO secretion or use of anti-IAPs reagents. The kinetics of the effect of TRAIL and Smac59 treatment on XIAP and cIAP-1 expression in HNSCC cell lines was investigated. UMSCC11B and HSC3M3 cells were seeded in 12 well plates, incubated for 24 hours at 37ºC and 5%CO_2. The medium was replaced with a fresh medium containing TRAIL (50ng/ml) or Smac59 (50nM). After 2, 4, 8 and 12hours of incubation, the cells were lysed and subjected to western blot analysis to investigate XIAP and cIAP-1 expression.

The results shown in Figure 5.7 indicated that in the UMSCC11B cell line, TRAIL induced XIAP and cIAP-1 downregulation at 8 hours and Smac59 induced cIAP-1 depletion as early as 2hours after treatment. Smac59 had no noticeable effect on XIAP expression in UMSCC11B. Similarly in the HSC3M3 line, Smac59 induced cIAP-1 depletion as early as 2hours after treatment and downregulated XIAP at 8hours. TRAIL had no effect on XIAP or cIAP-1 expression in HSC3M3 cell lines. Smac59 induced cIAP-1 depletion in both sensitive and resistant cell lines suggesting that cIAP-1 depletion may not be an indicator of Smac59 sensitivity. XIAP downregulation was observed in the sensitive cells when treated with the corresponding drug. Whether XIAP downregulation is a primary effect in TRAIL/Smac59 induced apoptosis or is a secondary event for apoptosis needs to be further investigated.

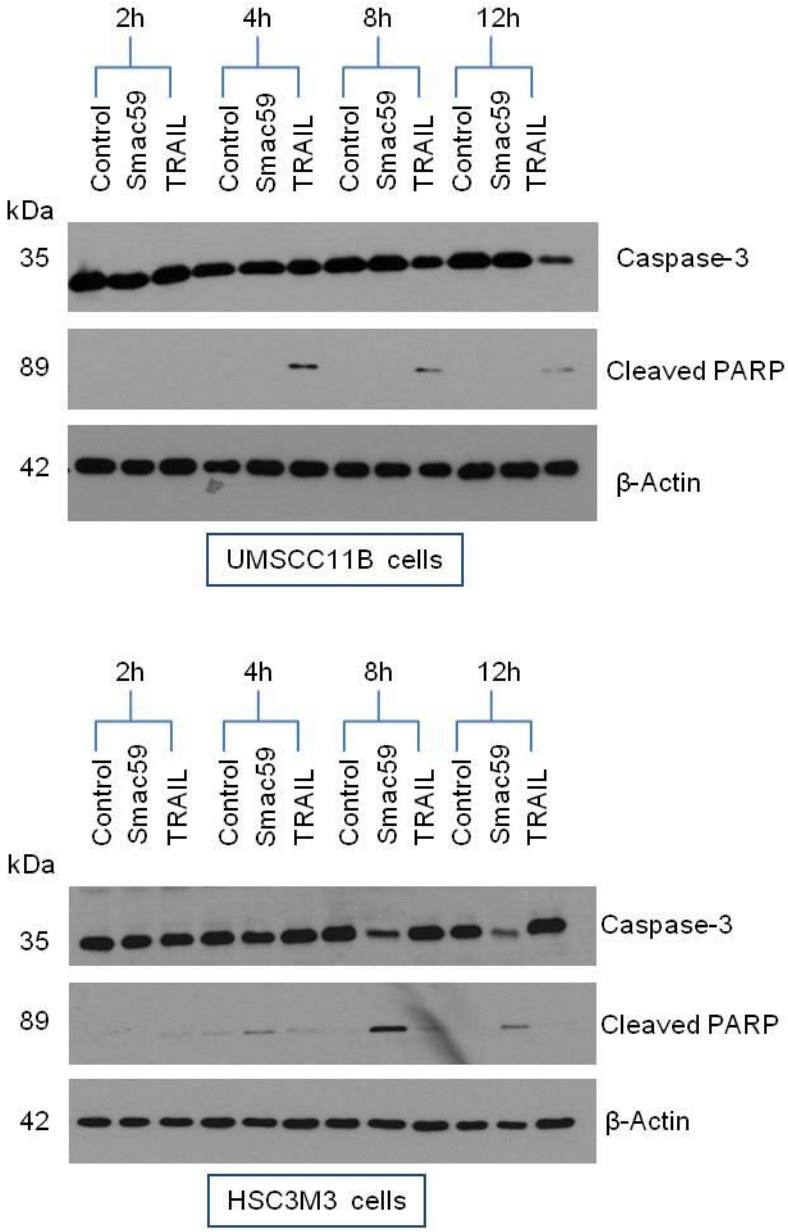


Figure 5.6 TRAIL and Smac59 induce caspase dependant apoptosis

HSC3M3 and UMSCC11B cells were seeded in 12 well plates, incubated for 24hours at 37ºC and 5%CO_2. The medium was replaced by a fresh medium containing TRAIL (50ng/ml) or Smac59 (50nM). After 2, 4, 8 and 12hours of incubation, the cells were lysed and subjected to western blot analysis to examine capase-3 activation and Poly (ADP-ribose) polymerase (PARP) cleavage. β-actin was used as a loading control.

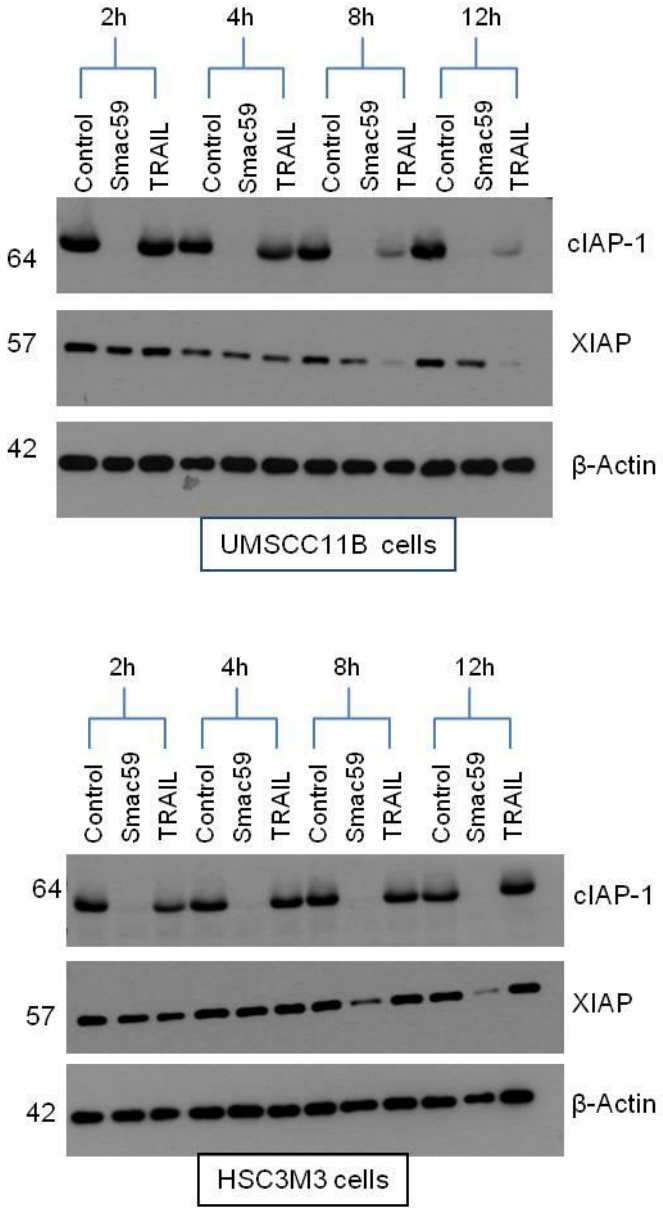


Figure 5.7 Effect of TRAIL and Smac59 on XIAP and cIAP-1 expression in HNSCC cells

HSC3M3 and UMSCC11B cells were seeded in 12 well plates, incubated for 24hours at 37ºC and 5%CO_2. The medium was replaced by a fresh medium containing TRAIL (50ng/ml) or Smac59 (50nM). After 2, 4, 8 and 12hours of incubation, the cells were lysed and subjected to western blot analysis to examine XIAP and cIAP-1 expression. β-actin was used as a loading control.

5.3.3 Effect of Caspase inhibitor treatment on IAPs (XIAP and cIAP-1) expression

The results obtained from the previous two experiments suggested that TRAIL and Smac59 induced caspase dependant apoptosis in HNSCC cell lines and that they induced downregulation of XIAP and cIAP-1. In order to investigate the nature of IAPs role in TRAIL and Smac59 induced apoptosis and whether the downregulation observed after TRAIL/Smac59 treatment is important for apoptosis induction or is a consequent event of apoptosis, UMSCC11B and HSC3M3 cells were seeded in 12well plates, incubated for 24hours at 37ºC and 5%CO_2. The medium was replaced by a fresh medium containing Pan-caspase inhibitor ZVAD-FMK (20µM). The cells were incubated for one hour. Then TRAIL (50ng/ml) or Smac59 (50nM) were added to the cells. The cells were then incubated for a further 8hours. The cells were lysed and subjected to western blot analysis to investigate XIAP and cIAP-1 expression.

The results shown in Figure 5.8 indicated that in the HSC3M3 cell line, caspase inhibitor was unable to block cIAP-1 depletion induced by Smac59 but it did inhibit XIAP downregulation. Similarly in the UMSCC11B cell line, pre-treatment with caspase inhibitor blocked XIAP downregulation. This data suggested that Smac59-induced cIAP-1 depletion was caspase independent and did not correlate with the sensitivity of HNSCC cell lines to Smac59. XIAP downregulation in UMSCC11B and HSC3M3 cells following TRAIL and Smac59 treatment respectively seemed to be caspase dependent. However, it is not clear from these experiments whether initiator caspases or executioner caspases might be responsible for blocking XIAP downregulation. To determine this, the effect of caspase type-specific inhibitors needs to be tested.

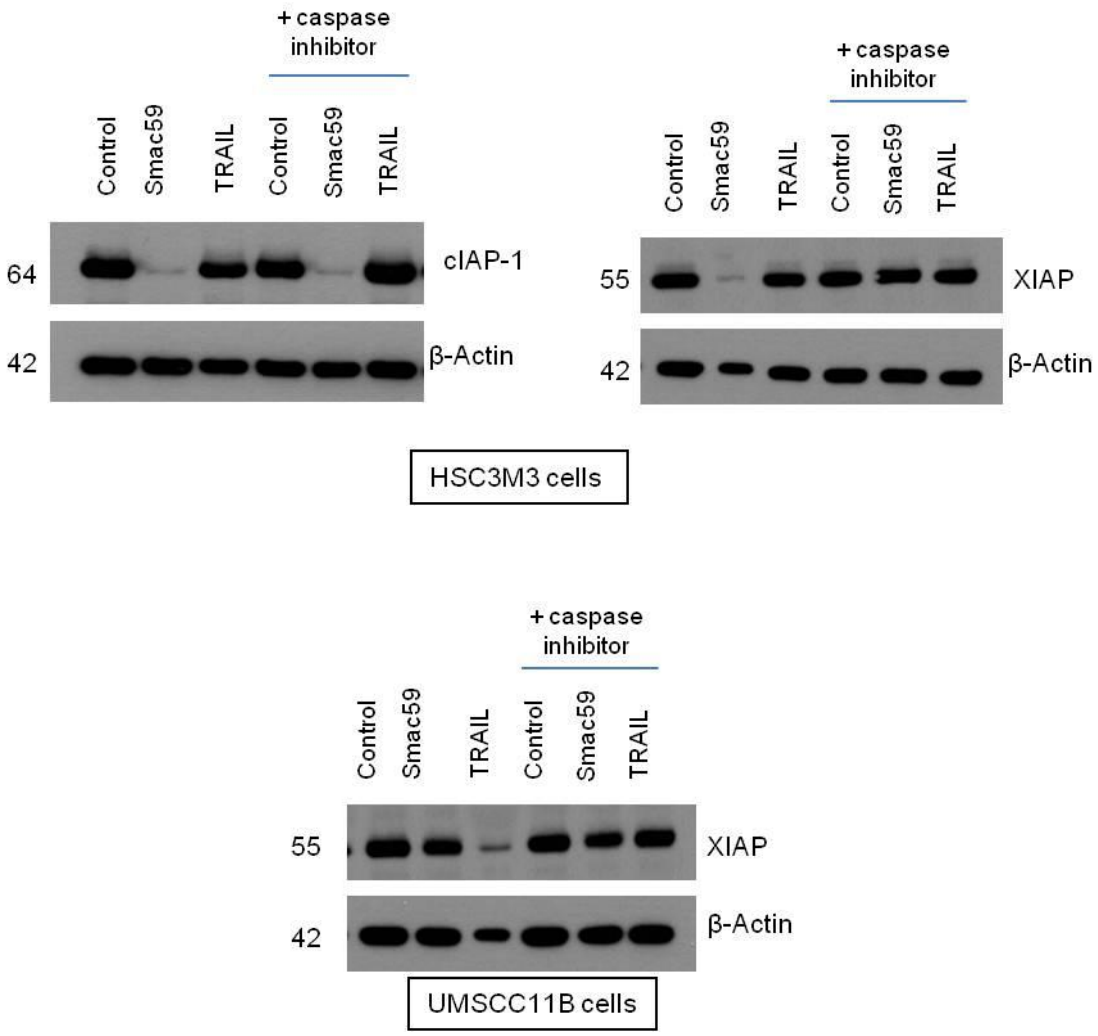


Figure 5.8 Effect of caspase inhibitor on IAPs expression with TRAIL/Smac59 treatment

HSC3M3 and UMSCC11B cells were seeded in 12well plates, incubated for 24hours at 37ºC and 5%CO_2. The medium was replaced by Pan-caspase inhibitor ZVAD-FMK (20µM). The cells were incubated for one hour. Then TRAIL (50ng/ml) or Smac59 (50nM) were added to the cells. The cells were then incubated for further 8 hours. The cells were lysed and subjected to western blot analysis to examine XIAP and cIAP-1 expression. β-actin was used as a loading control.

5.3.4 Effect of XIAP knockdown on TRAIL and Smac59 induced apoptosis

In order to understand the role of XIAP in TRAIL/Smac59 induced apoptosis, we investigated the effect of XIAP knockdown on TRAIL and Smac59 sensitivity in HNSCC cell lines. This was performed using two different approaches. The first one was to inhibit XIAP using siRNA. UMSCC11B and HSC3M3 cells were plated in 24 well plates and incubated for 24hours at 37ºC and 5% CO_2. The cells were transfected with XIAP-siRNA (5' AUCCAUCCAUGGCAGAUUA 3') using X-tremeGENE HP siRNA Reagent (Roche). The cells were incubated for 24 or 48hours. The cells were lysed and subjected to western blotting to test the gene knockdown (Figure 5.9A). The maximum XIAP knockdown was observed at 48 hours after transfection.

UMSCC11B and HSC3M3 cells were plated in 96 well plates and incubated for 24hours at 37ºC and 5%CO_2. Then the cells were transfected with XIAP-siRNA or scrambled siRNA. After 48hours the cells were treated with TRAIL (50ng/ml) or Smac59 (50nM) for 24hours and cell viability was tested using MTT assay. The results shown in Figure 5.9 B indicated that XIAP knockdown had undetectable effect on the sensitivity of UMSCC11B to Smac59 suggesting that XIAP knockdown is not sufficient to sensitise the resistant cells to Smac59. In HSC3M3 cells, XIAP knockdown increased their sensitivity to Smac59 but it had no effect on their sensitivity to TRAIL.

The second approach was to establish stable XIAP knockdown cells. UMSCC11B and HSC3M3 cells were plated in 24well plates and incubated for 24hours at 37ºC and 5%CO_2. Then the cells were transfected with PLenti6-sh-XIAP or PLenti6-sh-lacZ as an empty vector. The transfection was performed using X-tremeGENE HP DNA Transfection reagent. After 24hours, the medium was replaced with fresh medium containing the selectable marker Blasticidin (10ug/ml for UMSCC11B and 5ug/ml for HSC3M3). The cells were incubated for 1-2 weeks and medium was changed every 2 days. Then the mixed population of cells were tested for XIAP knockdown using western blotting. We were able to establish stable

XIAP knockdown from UMSCC11B cells but not from HSC3M3 cells (Figure 5.10).

In order to investigate the effect of stable XIAP knockdown on HNSCC cell lines, UMSCC11B, UMSCC11B-sh-lacZ and UMSCC11B-sh-XIAP cells were plated in 96well plates and incubated for 24hours at 37ºC and 5%CO_2. Then the cells were treated with TRAIL (50ng/ml) or Smac59 (50nM) for 24hours and cell viability was tested using MTT assay. The results shown in Figure 5.10 indicated that XIAP knockdown had no effect on the sensitivity of UMSCC11B to Smac59 and that XIAP knockdown could not sensitise UMSCC11B cells to Smac59-induced apoptosis. These data was consistent with the results obtained from the siRNA knockdown experiment.

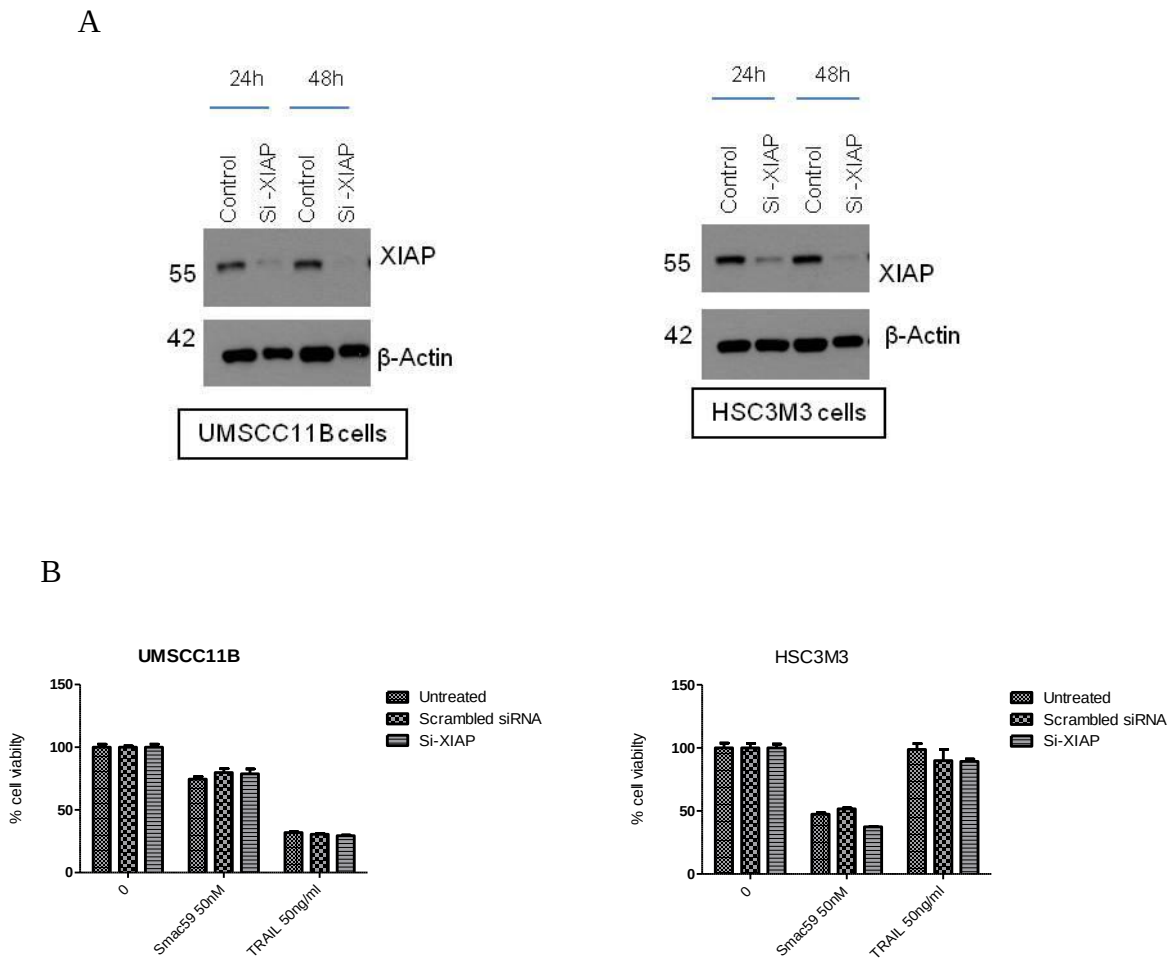


Figure 5.9 Effect of XIAP knockdown using siRNA on TRAIL/Smac59 sensitivity

A: HSC3M3 and UMSCC11B cells were seeded in 24well plates and incubated for 24hours at 37ºC and 5%CO_2. Then the cells were transfected with XIAP-siRNA using X-tremeGENE HP siRNA Reagent. The cells were incubated for 24 or 48hours and gene knockdown was tested using western blotting. β-actin was used as a loading control B: UMSCC11B and HSC3M3 cells were plated in 96well plates and incubated for 24hours at 37ºC and 5%CO_2. Then the cells were transfected with XIAP-siRNA or scrambled siRNA. After 48hours the cells were treated with TRAIL (50ng/ml) or Smac59 (50nM) for 24hours then cell viability was tested using MTT assay. The results are shown as the percentage of the viable cells after treatment with respect to viable non treated cells. All experiments were performed in triplicate, error bars indicate SD.

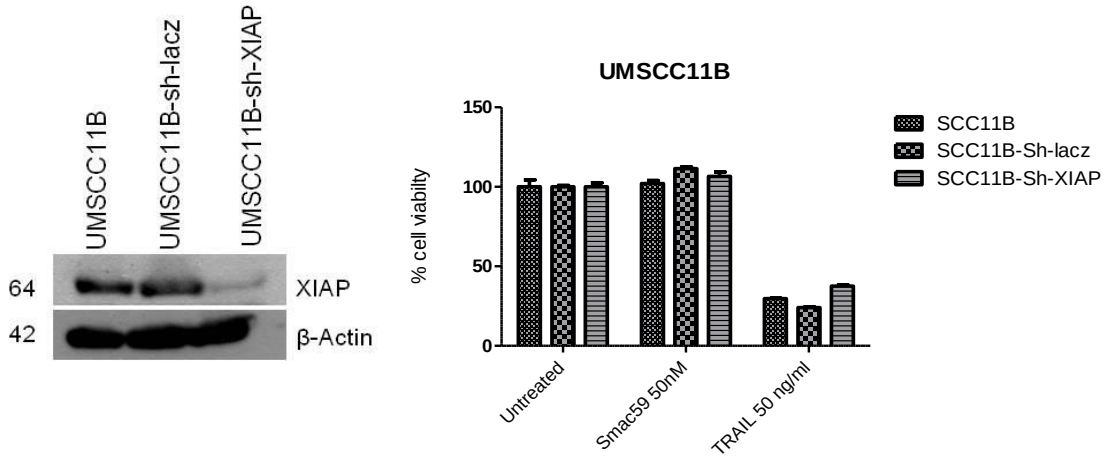


Figure 5.10 XIAP knockdown stable clones

Left: UMSCC11B cells were plated in 24well plates and incubated for 24hours at 37ºC and 5%CO_2. Then the cells were transfected with PLenti6-sh-XIAP or PLenti6-sh-lacZ using X-tremeGENE HP DNA Transfection reagent. The following day, the medium was replaced with fresh medium containing the selection antibiotic Blasticidin (10 and 5ug/ml). The cells were incubated for 1-2 weeks and medium was changed every 2days. Then the cells were tested for XIAP knockdown using western blotting. β-actin was used as a loading control. Right: UMSCC11B-sh-XIAP cells were seeded in 96well plates, incubated for 24hours at 37ºC and 5%CO_2. Then TRAIL (50ng/ml) or Smac59 (5 nM) were added to the cells, incubated for 24 hours then cell viability was tested using MTT assay. The results are shown as the percentage of the viable cells after treatment with respect to viable non treated cells. All experiments were performed in triplicate, error bars indicate SD.

In conclusion, the results of this chapter suggested that:

- Smac59 increased TNF-α autocrine secretion in the Smac59 sensitive (TRAIL resistant) HNSCC cell lines.
- Recombinant TNF-α was able to sensitise Smac59 resistant (TRAIL sensitive) HNSCC cells to Smac59.
- TNF-α inhibition had no significant effect on Smac59 induced apoptosis in Smac59 sensitive HNSCC cells.
- Bcl-2 overexpression increased resistance to TRAIL in HNSCC cell lines, however it showed undetectable effect on Smac59 sensitivity.
- TRAIL and Smac59 induced a caspase dependent cell death in HNSCC cell lines.
- TRAIL and Smac59 induced XIAP downregulation in sensitive HNSCC cell lines. This down regulation was inhibited with pan-caspase inhibitor.
- XIAP knockdown could not sensitise TRAIL or Smac59 resistant cells, however it was able to increase the sensitivity of Smac59 cells to Smac59.

6 Results IV

Biomarkers of treatment outcome in head and neck squamous cell carcinoma.

Treatment of cancer patients with conventional therapies normally is successful only initially. However, in many patients tumour recurs and patients relapse, such tumours exhibit the resistant phenotype to various cytotoxic and apoptotic agents. Cancer cells can become resistant to radio-chemotherapy by developing mechanisms to repair DNA damage or decreasing the uptake of drugs across the cell membrane (Lee et al., 2007). To avoid the resistance and adverse effects of different therapeutics, it is essential to characterise individual tumour specimens to establish a genetic profile prior to each patient treatment which may predict response to a given treatment. For this to become possible in routine clinical practice, biomarkers and appropriate sensitivity assays need to be employed to reliably identify those patients who may benefit from a certain treatment. The need for specific and effective therapy is essential as the survival rate of HNSCC is low with high mortality rate and finding such therapy will be the optimum achievement.

ERCC1, HIF-1α, Ku80, PTEN, Rad51 and XRCC1 proteins are involved in the DNA damage repair and tumour hypoxia. Their expression has been associated with resistance to radiotherapy and/or chemotherapy and with the survival of patients with different types of tumour as discussed in the introduction. HPV positive carcinomas showed better response to treatment and overall survival.

In this chapter, we investigated the immuoexpression of ERCC1, HIF-1α, Ku80, PTEN, Rad51 and XRCC1 proteins in HNSCC tissues. Furthermore, we investigated the association between their levels of expression and patient survival and response to treatment. In addition, we investigated the HPV status in the tonsil and pharyngeal carcinomas and its association with survival and expression of the above mentioned proteins was analyzed.

6.1 Patients and carcinomas

Tissue biopsies from head and neck squamous cell carcinomas (HNSCC) were obtained from the pathology archives of the Oral Pathology Department, Dental Institute, King's College London from patients treated in the period 1997 to 2004. The cases were selected to be representative of a range of oral and pharyngeal sub-sites. Table 6.1 shows the number of cases for each sub-site. Details of clinical and histopathological features and the follow up information for each case were collected from pathology records, clinical notes and various hospital clinical management databases. Data were recorded in Excel spreadsheet before being exported to IBM SPSS statistics 20 program for analysis. The data recorded was site of origin, age at the time of diagnosis, gender, tumour differentiation, TNM stage (6^{th} edition), treatment (surgery or surgery followed by radiotherapy), disease free survival and overall survival. The overall survival was calculated in months from the date of initial surgery to the date of last follow-up or death, and the disease free interval was calculated from the date of initial surgery to the date of first recurrence or the date of last follow-up without evidence of recurrent or persistent disease. The ethics approval for this study was obtained from Guy's Hospital Research Ethics Committee, reference 04/02/10).

Table 6.1 Sub-site distribution of the study cases

Site	Number of cases
Lateral tongue	27
Buccal mucosa	17
Retromolar, tuberosity	19
Floor of the mouth and palate	17
Alveolus	18
Lip vermillion	17
Tonsils	20
Pharynx	15
Total	150

6.1.1 Clinical and pathological characterization

The median age of the 150 patients at diagnosis was 60 years (range, 26 to 91 years). 99 (66%) patients were male and 51 (34%) patients were female. Histologically, 23 (15.3%) cases were well differentiated carcinoma, 79 (52.7%) cases were moderately differentiated and 48 (32.0%) cases were poorly differentiated. Using the TNM staging system 6th edition, 21 (14%) cases were stage I, 32 (21.3%) cases were stage II, 27 (18%) cases were stage III and 70 (46.7%) cases were stage IV. No metastasis was present in 85 (56.7%) cases while 65 (43.3%) cases had metastasized to regional lymph nodes at diagnosis. 78 (52%) cases were treated by surgery only and 69 (46%) cases were treated with surgery followed by radiotherapy with curative intent, 3 (2%) cases had palliative therapy. This data broken down by sub-site is shown in Table 6.2.

For all sites of origin, males were affected more frequently than females. Moderate tumour differentiation was most common except for tonsil and pharynx in which poorly differentiated tumours were more common, though in at least some cases the undifferentiated type may refer a differentiated carcinoma of tonsil crypt epithelium as HPV associated carcinoma of tonsil was not recognized at the time of original diagnosis. Metastasis to regional lymph nodes was observed in cases from all sites but was more frequent in lateral tongue cases. Carcinoma of the lip cases was the least likely to metastasize. TNM stage IV was predominant in carcinomas from the lateral tongue, alveolus, tonsils and retromolar region, tuberosity and palate. Stage I was predominant in lip cases. Stage III and stage IV were equally distributed in cases of floor of the mouth. Stage II and Stage IV were comparable in buccal mucosa cases. This data is summarised by site in Figure 6.1and Figure 6.2.

The diagnostic tissue specimens from the 150 carcinomas were used to construct tissue microarray (TMA) blocks as described in the materials and methods section 2.2.14. 14 specimens were post-radiotherapy tissues. This was discovered after array construction and all data from these cases has been excluded from biomarker analysis of radio-resistance leaving 136 for this analysis.

Table 6.2 Clinical and histopathological characteristics of the study cases and carcinomas

	Number of cases (%)								
Characteristic	**Lateral Tongue**	**Buccal mucosa**	**Retro-palate**	**Floor of mouth**	**Alveolus**	**Lip**	**Tonsil**	**Pharynx**	**Total**
Age, median, range	58,(26-91)	59, (37-81)	63, (41-87)	60, (44-85)	64, (39-85)	73, (40-83)	52, (38-88)	62, (45-74)	60, (26-91)
Sex									
Female	12 (44.4%)	6 (35.3%)	8 (42.1%)	5 (29.4%)	7 (38.9%)	4 (23.5%)	2 (10%)	7 (46.7%)	51 (43%)
Male	15 (55.6%)	11 (64.7%)	11 (57.9%)	12 (70.6%)	11 (61.1%)	13 (76.5%)	18 (90%)	8 (53.3%)	99 (66%)
Differentiation									
Well	8 (29.6%)	3 (17.6%)	3 (15.8%)	0 (0%)	1 (5.6%)	6 (35.3%)	2 (10%)	0 (0.0%)	23 (15.3%)
Moderate	11(40.7%)	13 (76.5%)	11 (57.9%)	12 (70.6%)	14 (77.8%)	10 (58.8%)	3 (15%)	5 (33.3%)	79 (52.7%)
Poor	8 (29.6%)	1 (5.9%)	5 (26.3%)	5 (29.4%)	3 (16.7%)	1 (5.9%)	15 (75%)	10 (66.7%)	48 (32.0%)
TNM stage									
I	3 (11.1%)	3 (17.6%)	0 (0%)	2 (11.8%)	3 (16.7%)	8 (47.1%)	1 (5%)	1 (6.7%)	21 (14%)
II	6 (22.2%)	6 (35.3%)	5 (26.3%)	3 (17.6%)	2 (11.1%)	4 (23.5%)	4 (20%)	2 (13.3%)	32 (21.3%)
III	5 (18.5%)	2 (11.8%)	2 (10.5%)	6 (35.3%)	2 (11.1%)	3 (17.6%)	2 (10%)	5 (33.3%)	27 (18%)
IV	13 (48.1%)	6 (35.3%)	12 (63.2%)	6 (35.3%)	11 (61.1%)	2 (11.8%)	13 (65%)	7 (46.7%)	70 (46.7%)
Lymph node metastasis									
No	11 (40.7%)	14 (82.4%)	14 (73.7%)	6 (35.3%)	9 (50%)	16 (94.1%)	8 (40%)	7 (46.7%)	85 (56.7%)
Yes	16 (59.3%)	3 (17.6%)	5 (26.3%)	11 (64.7%)	9 (50%)	1 (5.9%)	12 (60%)	8 (53.3%)	65 (43.3%)
Treatment									
Surgery	12 (44.4%)	12 (75%)	17 (94.4%)	5 (29.4%)	8 (44.4%)	13 (81.2%)	5 (25%)	6 (40%)	78 (53.1%)
Surgery + radiotherapy	15 (55.6%)	4 (25%)	1 (5.6%)	12 (70.6%)	10 (55.6%)	3 (18.8%)	15 (75%)	9 (60%)	69 (46.9%)

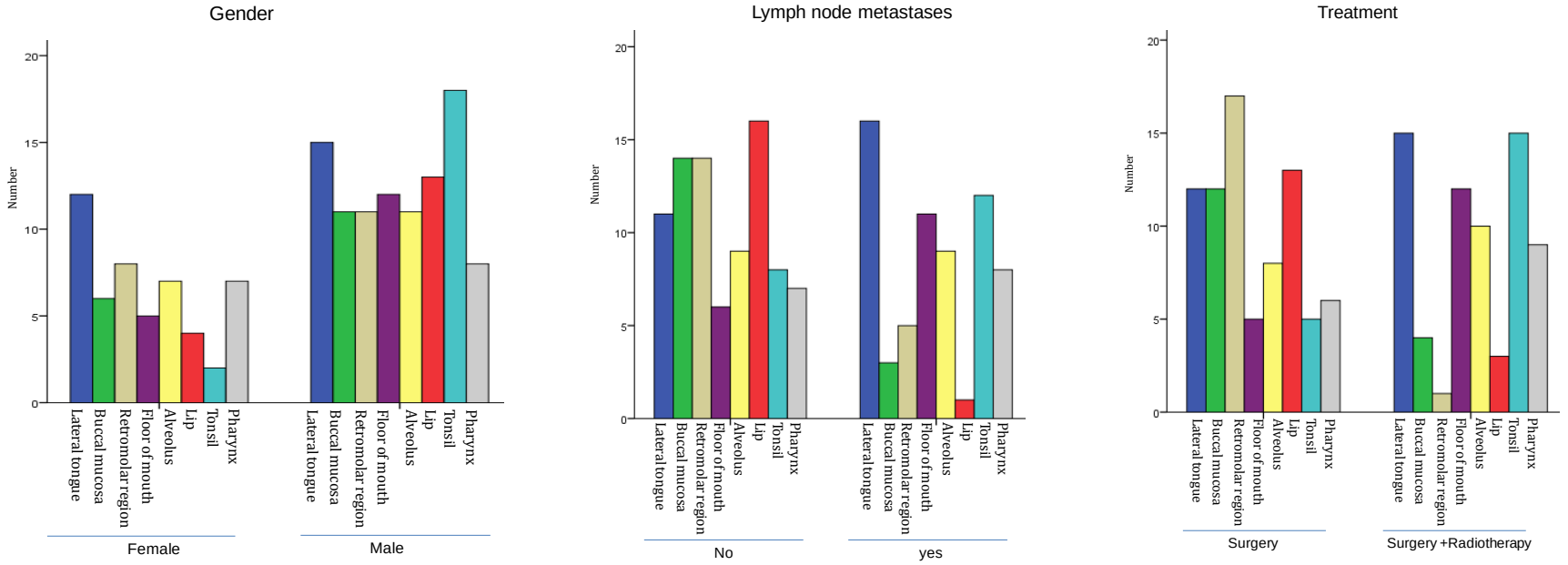


Figure 6.1 Clinical and histopathological characteristics of study cases and carcinomas

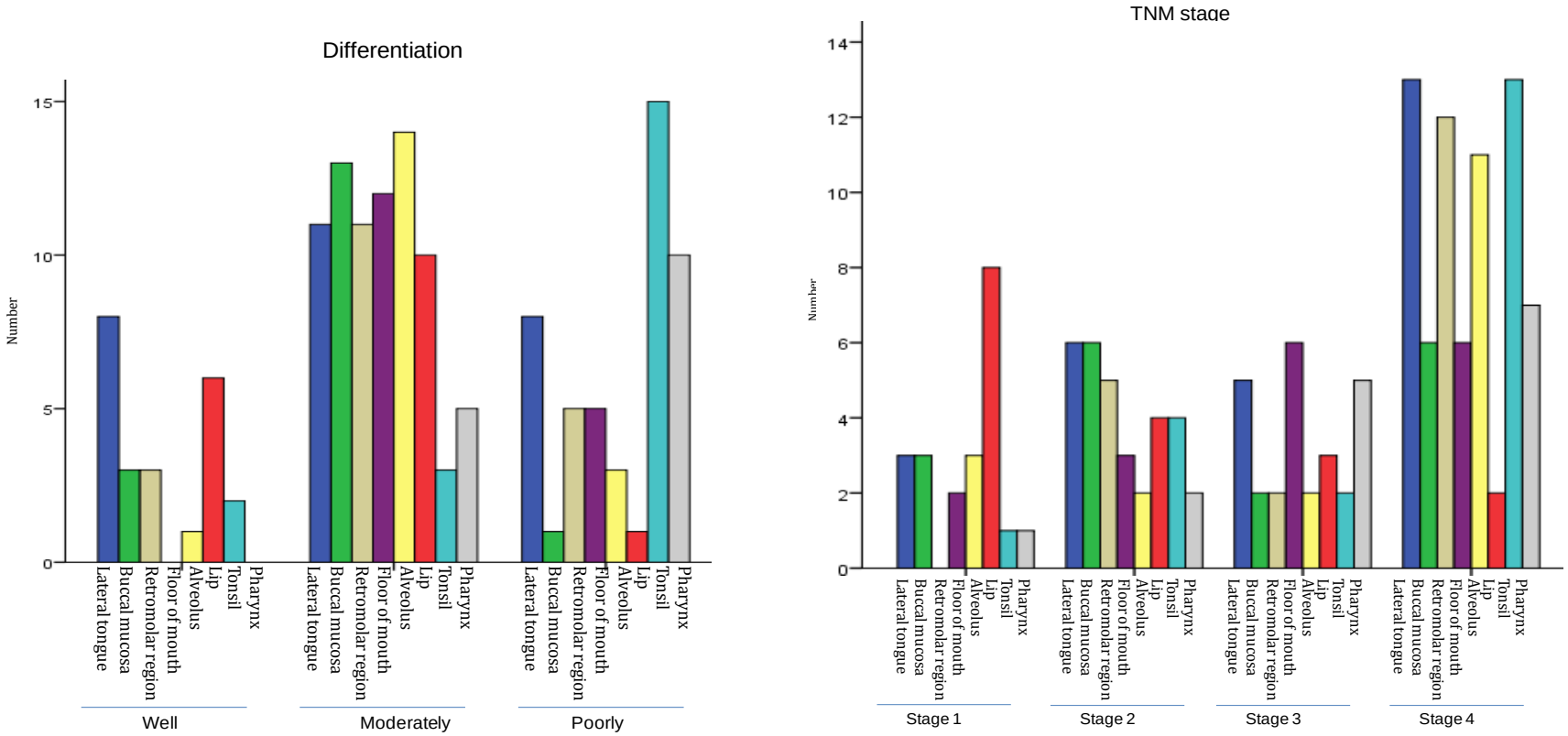


Figure 6.2 Clinical and histopathological characteristics of study cases and carcinomas

6.2 Immunohistochemical staining

Paraffin embedded formalin fixed (PEFF) tissue specimens from 150 cases were used to construct tissue microarray (TMA) blocks for immunostaining as described in materials and methods section 2.2.14. Immunohistochemical staining was performed to examine the expression of ERCC1, HIF-1α, KU80, PTEN, Rad51 and XRCC1 using the appropriate antibody. Antigen retrieval, antibody concentration and incubation period and use of positive controls were optimised for each antibody.

TMA blocks were sectioned at 4μm using routine histological procedures on a Leica-RM2235 microtome by staff in the KCL Department of Oral Pathology and stored for a maximum of three days before staining. Two sections from each TMA were stained as described in the material and methods section 2.2.15. Images of IHC staining were taken using a Zeiss microscope with an Olympus CV3 camera and Olympus Cell-D software.

The expression of each antibody was scored by consensus between two pathologists (Odell E. and El-Attar R.H.) blind to clinical and pathological data. Two parameters were scored for each antibody, the intensity of the staining (1=no stain, 2=equivocal, 3=weak, 4=moderate or 5=strong) and the percentage of the stained cells (1=0-25%, 2=26-50%, 3=51-75% or 4=76-100%). Both assessments were subjective. Four carcinoma cores were scored in duplicate for each case and a final combined score was calculated as the average of the eight scores.

The scores were recorded in the spreadsheet with the clinical and the histological data for each case and imported into the IBM SPSS statistics 20 programme for statistical analysis. Due to limited number of cases in each band of the scoring system used and to increase statistical power, scores from each parameter were transformed into a binary score. Intensity of the staining was transformed to either negative (which included 1=no stain, 2=equivocal) or positive (which included 3=weak, 4=moderate and 5=strong). Percentage of cells stained was transformed to either low (which included 1=0-25%) or high (which included

2=26-50%, 3=51-75% and 4=76-100%). These combinations were selected on the basis of score distribution. When less than 25% of cells were positive, the number of cells positive was usually very low, so that there appeared to be a natural division into two groups.

6.2.1 ERCC1 Immunostaining

The immunostaining for ERCC1 was nuclear in normal, dysplastic and carcinoma tissue. ERCC1 positivity was variable in intensity and distribution in carcinoma tissues. When the staining was strong, it usually involved all carcinoma cells except the keratinizing ones. When the staining was weak, it was focally distributed involving mainly the immature cells. Suprabasal staining with little basal positivity was observed in normal and dysplastic epithelium. Stromal cells showed weak positive staining. Minimal background staining was observed in some cases, but the reaction overall appeared strong (Figure 6.3).

6.2.2 HIF-1α immunostaining

Staining for HIF-1α was nuclear with variable intensity and distribution. Mostly the maturing cells showed positive staining (Figure 6.3). Occasionally stromal cells showed positive staining. Normal epithelium and dysplasia showed suprabasal positivity. Background staining was observed in some cases.

6.2.3 Ku80 immunostaining

Ku80 staining was nuclear, well defined with no background staining. All carcinoma tissues expressed Ku80 to different degrees. Staining intensity was moderate to strong. Mature cells showed positive attaining. Stromal cells were positive for ku80 (Figure 6.4).

6.2.4 PTEN immunostaining

PTEN immunostaining was observed to be both nuclear and cytoplasmic, only nuclear staining was scored. The immature basal and suprabasal cells were

positive. Mature and keratinizing cells were negative. Stromal cells showed strong positivity. The overall carcinoma positive staining was weak with a few cases which showed strong staining intensity. PTEN expression was usually observed in small numbers of cells (Figure 6.4).

6.2.5 Rad51 immunostaining

Rad51 staining was nuclear with some cases showed strong cytoplasmic staining. Mostly suprabasal cells were positive. Stromal and muscle cells showed strong positivity. Background staining was observed in most of the cases when antibody was incubated overnight but staining was faint when incubated for 30 min as recommended by manufactures, so optimization for anti-Rad51 was modified to 3 hours incubation period to balance staining intensity in carcinoma cells with minimal background staining (Figure 6.5).

6.2.6 XRCC1 immunostaining

XRCC1 staining was mainly nuclear but a few cases showed cytoplasmic staining. Almost all HNSCC tissues were positive to XRCC1 staining with strong intensities. Positive staining was confined to proliferating basal and suprabasal cells. Stromal cells showed positive staining in some cases. Minimal background staining was observed in a few cases (Figure 6.5).

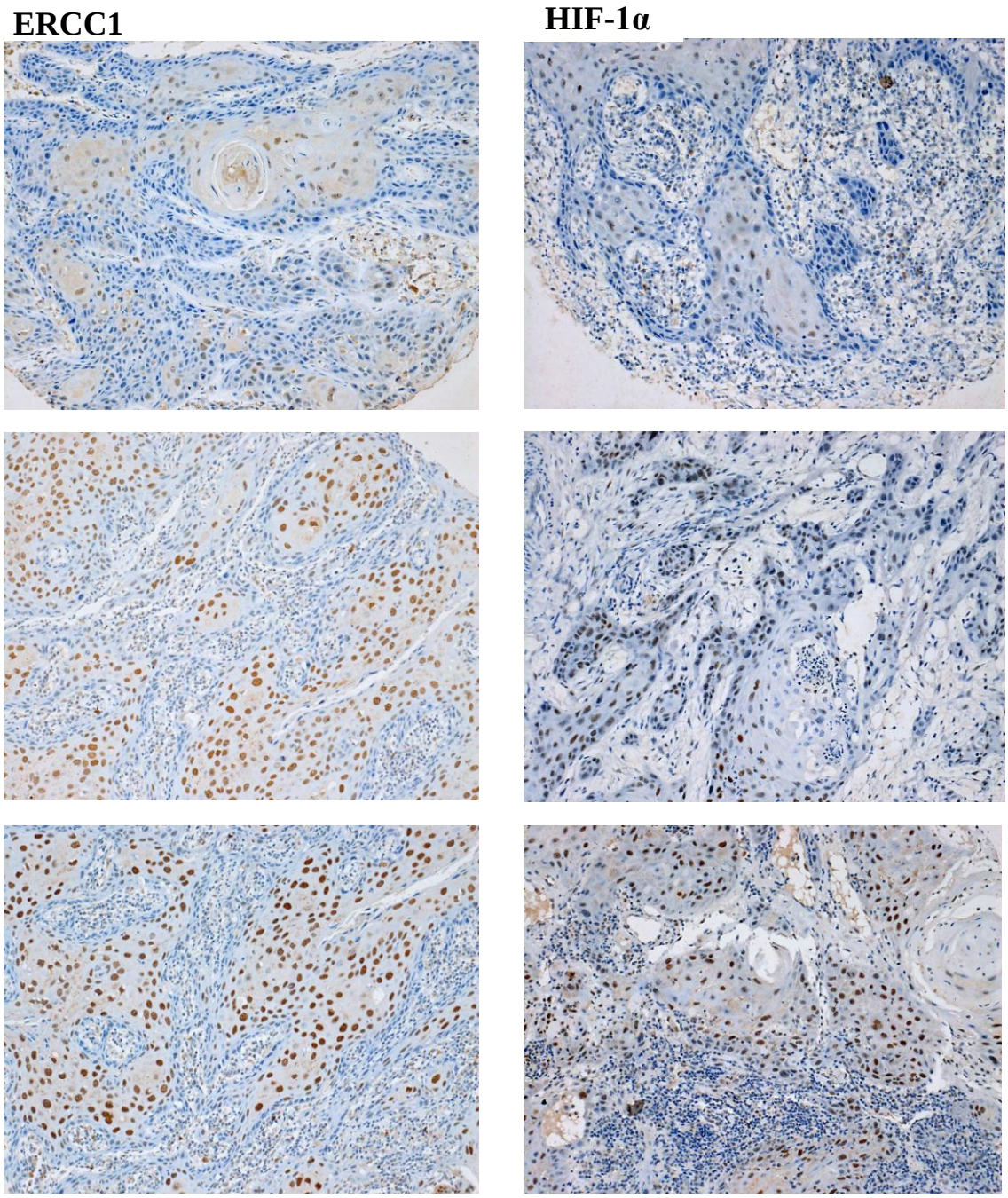


Figure 6.3 ERCC1 and HIF-1α expression in HNSCC tissues

Photomicrographs showing representative immunostaining for ERCC1 and HIF-1α expression in HNSCC tissues. The staining was nuclear with variable intensity. Original magnification10x objective, direct to 5 Mpixel camera.

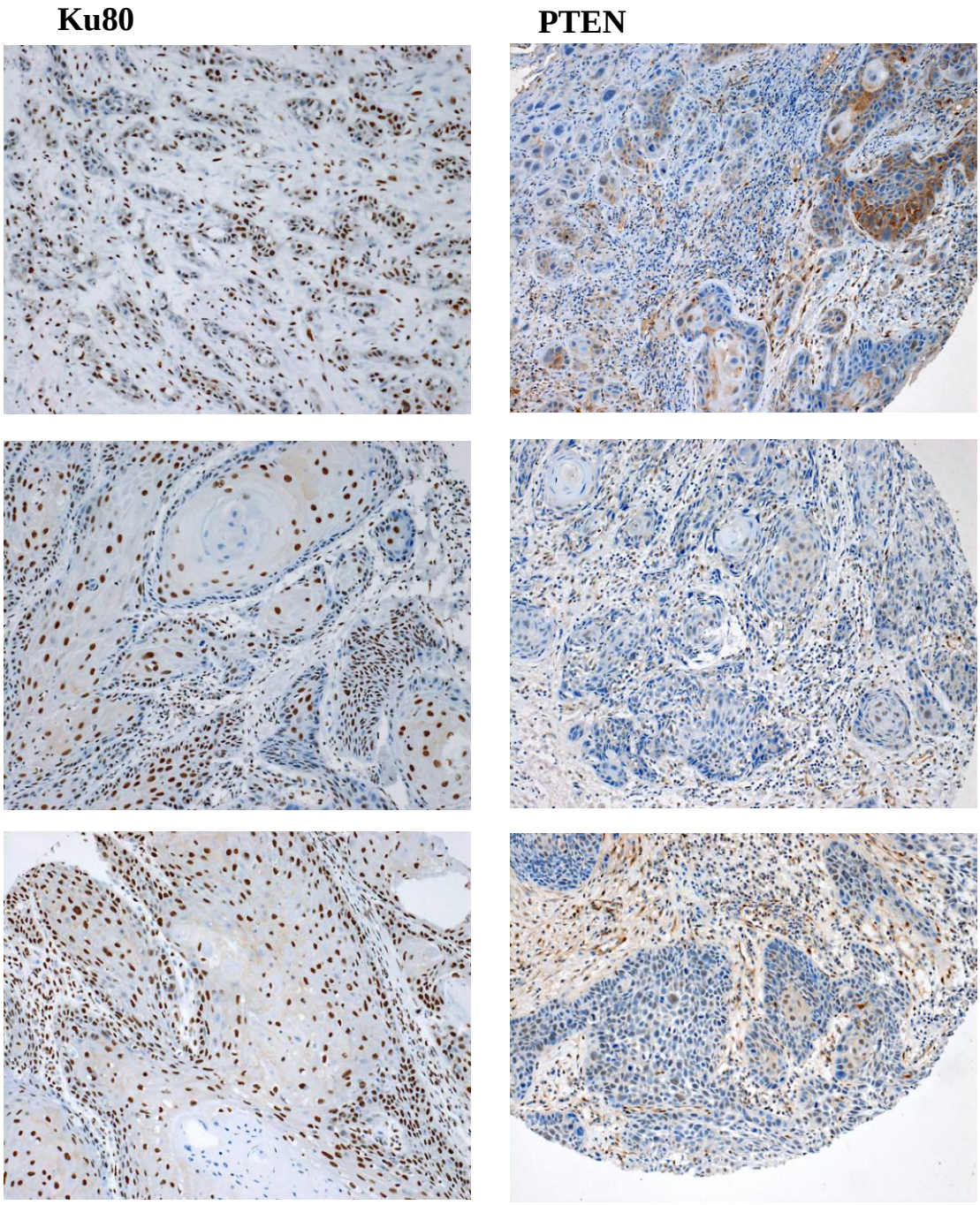


Figure 6.4 Ku80 and PTEN expression in HNSCC tissues

Photomicrographs showing representative immunostaining for Ku80 and PTEN expression in HNSCC tissues. Ku80 staining was nuclear with variable intensity and PTEN staining was nuclear and cytoplasmic. Original magnification10x objective, direct to 5M pixel camera.

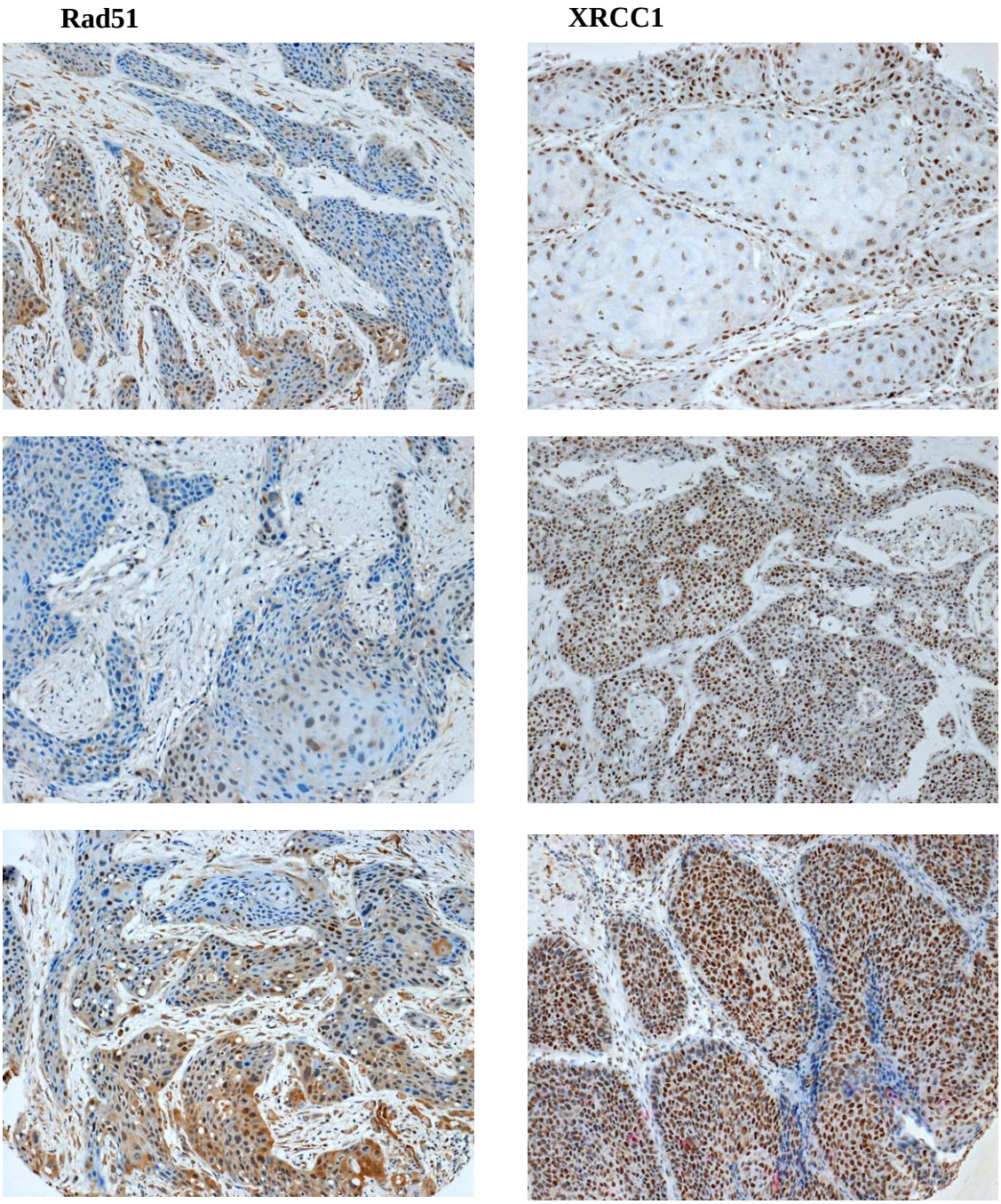


Figure 6.5 Rad51 and XRCC1 expression in HNSCC tissues

Photomicrographs showing representative immunostaining for Rad51 and XRCC1 expression in HNSCC tissues. Rad51 showed both nuclear and cytoplasmic staining with different intensities and distribution. XRCC1 staining was only nuclear nuclear with variable intensities. Original magnification10x objective, direct to 5M pixel camera.

6.3 Survival analysis

Clinical and pathological features of the study cases as well as the expression levels of ERCC1, HIF-1α, Ku80, PTEN, Rad51 and XRCC1 genes were tested for their influence on overall survival and disease free survival. Overall survival was calculated in months from the date of surgery until the date of death or date of last follow up. Disease free survival was calculated in months from date of surgery to the date of first recurrence or date of last follow up.

6.3.1 Clinical and histopathological features and survival

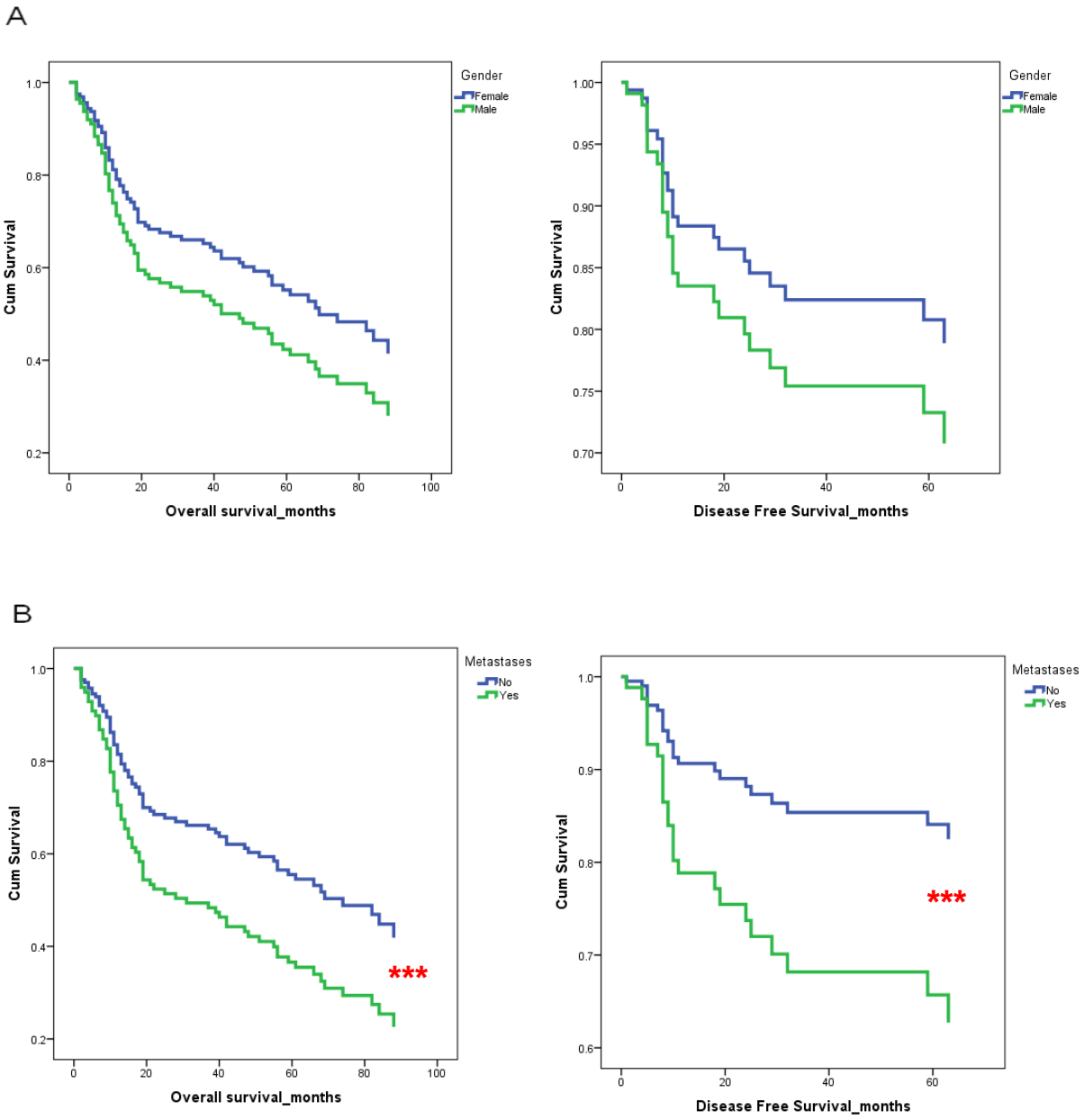
Overall survival and disease free survival in relation to gender, carcinoma site, tumour differentiation, TNM stage, metastasis to regional lymph nodes and treatment was analysed using Cox Regression analysis. All 150 cases were included in this analysis. Detailed *P* values, Odds Ratio (OR) and 95% confidence intervals (CI) are listed in Table 6.3.

- Males were approximately one and a half times more likely to die or develop recurrence than females (Figure 6.6A).
- Cases that metastasized to regional lymph node were significantly more likely to die or recur than cases with no detectable lymph node metastasis at time of diagnosis. *P v*alues for overall survival was *(P=0.026*) and for disease free survival (*P=0.034*) (Figure 6.6B).
- In relation to lateral tongue carcinomas, pharynx, floor of the mouth, lip carcinomas were more likely to kill the patient and tonsil, retromolar and palate, buccal mucosa and alveolus carcinomas were less likely to kill the patient. Carcinomas of floor of the mouth, lip and buccal mucosa were more likely to recur and tonsil, pharynx and alveolus carcinomas were less likely to recur. However, these differences was not significant statistically, except for disease free survival of floor of the mouth carcinomas (*p=0.019)* (Figure 6.7A).

- Tumour differentiation did not affect the overall survival of the patient. However, moderately and poorly differentiated carcinomas were more likely to recur than well differentiated carcinomas (Figure 6.7B).
- In relation to TNM stage1, cases at TNM stage 3 and stage 4 were more likely to die and stage 2 cases were less likely to die. Stage 3 carcinomas were more likely to recur and stage 2 carcinomas were less likely to recur (Figure 6.8A).
- Cases treated with surgery followed by radiotherapy were significantly more likely to die (P=0.064) and to recur (P=0.003) than patients treated with surgery only (Figure 6.8B).

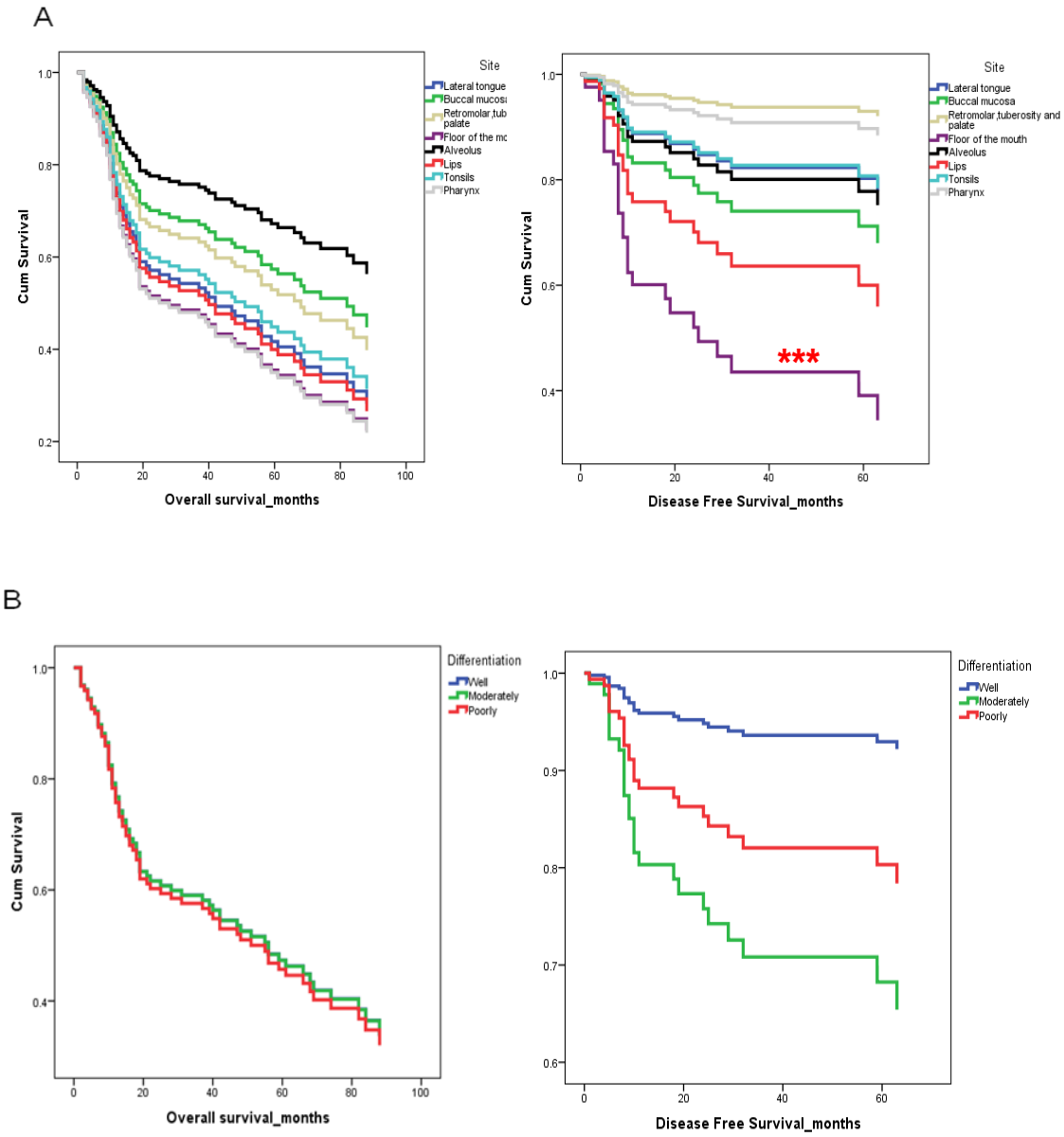
Table 6.3 *P* values, Odds Ratios (OR) and 95% confidence interval (CI) for the survival analysis of the study cases

	Overall survival			**Disease free survival**		
Variable	***P* value**	**OR**	**95% CI**	***P* value**	**OR**	**95% CI**
Gender	0.161	1.445	0.864-2.419	0.399	1.458	0.607-3.5
Lateral tongue	0.646			0.066		
Buccal mucosa	0.373	0.635	0.23-1.72	0.570	1.544	0.34-6.92
Retr-palate	0.441	0.727	0.32-1.63	0.322	0.331	0.03-2.96
Floor of mouth	0.685	1.183	0.52-2.66	**0.019**	4.274	1.27-14.38
Alveolus	0.121	0.454	0.16-1.23	0.862	1.142	0.25-5.11
Lip	0.934	1.047	0.33-3.11	0.334	2.324	0.42-12.89
Tonsils	0.822	0.916	0.42-1.96	0.473	0.474	0.21-4.35
Pharynx	0.648	0.648	0.54-2.62	0.528	0.494	0.05-4.42
Well	0.984			0.161		
Moderately	0.996	1.002	0.5-1.98	0.108	5.228	0.69-39.32
Poorly	0.897	1.048	0.51-2.13	0.304	3.00	0.36-24.38
Stage 1	0.167			0.115		
Stage 2	0.735	0.849	0.32-2.19	0.415	0.514	0.1-2.54
Stage 3	0.135	2.022	0.8-5.08	0.248	2.229	0.57-8.67
Stage 4	0.426	1.387	0.62-3.10	0.805	0.853	0.24-3.00
L.N metastasis	**0.026**	1.708	1.06-2.74	**0.034**	2.423	1.06-5.5
Treatment	0.064	1.583	0.97-2.57	**0.003**	5.095	1.74-14.88



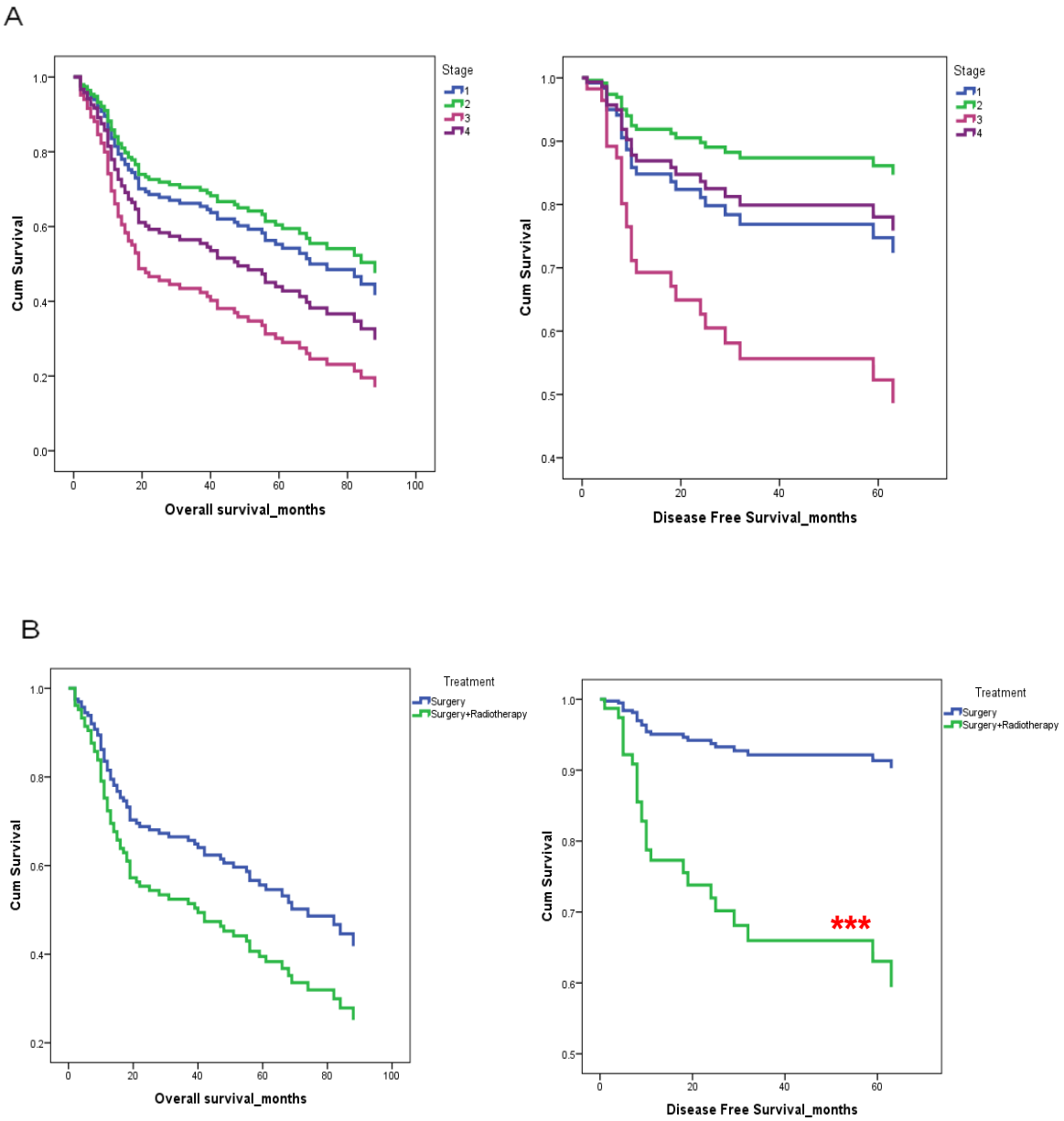
*** Statistically significant *P* value

Figure 6.6 Cox Regression survival analysis graphs of the study cases



*** Statistically significant *P* value

Figure 6.7 Cox Regression survival analysis graphs of the study cases



*** Statistically significant *P* value

Figure 6.8 Cox Regression survival analysis graphs of the study cases

6.3.2 DNA damage repair genes expression and survival

Overall survival and disease free survival in relation to expression level of ERCC1, HIF-1α, Ku80, PTEN, Rad51 and XRCC1 genes in HNSCC tissues were tested using Cox Regression analysis. The 14 post-radiotherapy specimens were excluded from this analysis. Detailed *P* values, Odd Ratios (OR) and 95% confidence interval (CI) are listed in Table 6.4.

- ERCC1 positive carcinomas were more likely to kill the patients and more likely to recur than ERCC1 negative carcinomas. Carcinomas with high percentage of ERCC1 expressing cells were more likely to recur than carcinomas with low percentage ERCC1 expressing cells (Figure 6.9).
- HIF-1α positive carcinomas and carcinomas with high percentage of HIF-1α expressing cells were more likely to kill the patient than HIF-1α negative or low expressing carcinomas. HIF-1α positive carcinomas and carcinomas with high percentage of HIF-1α expressing cells were significantly more likely to recur than HIF-1α negative or low expressing carcinomas (Figure 6.10).
- PTEN positive carcinomas were more likely to kill patients and to recur than PTEN negative carcinomas. Carcinomas with high percentage of PTEN expressing cells were significantly more likely to kill the patients and more likely to recur than carcinomas with low percentage of PTEN expressing cells (Figure 6.11).
- Carcinomas which expressed high Rad51were more likely to kill the patients and to recur than carcinomas with low Rad51expression (Figure 6.12).
- Carcinomas which expressed high XRCC1were more likely to kill the patients than carcinomas with low XRCC1expression (Figure 6.13).

- Ku80 high expressing carcinomas were more to kill the patient and to recur than Ku80 low expressing carcinomas (Figure 6.14).

Table 6.4 *P* values, Odds Ratios (OR) and 95% confidence interval (CI) for the survival analysis of the study cases

	Overall survival			**Disease free survival**		
Variable	***P* value**	**OR**	**95% CI**	***P* value**	**OR**	**95% CI**
ERCC1 intensity	0.52	1.18	0.694-2.037	0.225	1.981	0.65-5.98
ERCC1 %	0.94	0.47	0.389-2.423	0.175	2.35	0.68-8.07
HIF-1α intensity	0.40	1.23	0.753-2.032	**0.011**	4.997	1.45-17.16
HIF-1α %	0.68	1.60	0.966-2.674	**0.005**	3.657	1.46-9.12
Ku80 intensity	calculation of *p* value was not possible because all cases within the analysis were positive					
Ku80 %	0.20	3.55	0.49-25.65	0.492	21.848	0.003-146532
PTEN intensity	0.47	1.20	0.72-1.99	0.508	1.362	0.54-3.39
PTEN %	**0.042**	1.72	1.02-2.41	0.305	1.665	0.62-4.4
Rad51 intensity	0.80	0.94	0.57-1.54	0.488	0.993	0.39-2.47
Rad51 %	0.25	1.35	0.8-2.29	0.752	1.169	0.44-3.00
XRCC1 intensity	0.93	0.94	0.23-0.3.88	0.589	21.165	0.00-1364598
XRCC1 %	0.38	1.45	0.62-3.38	0.251	25.636	0.1-649773

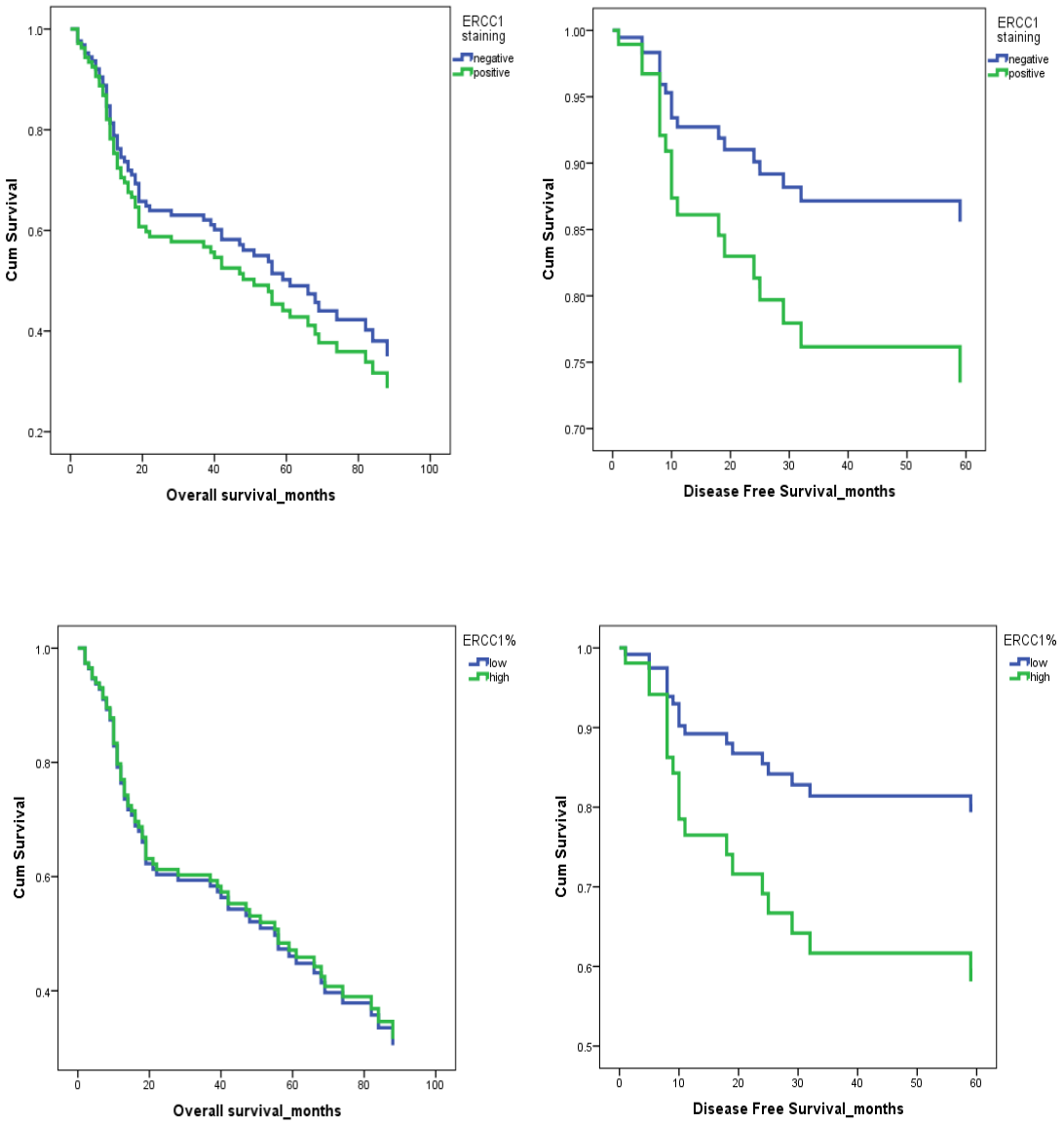
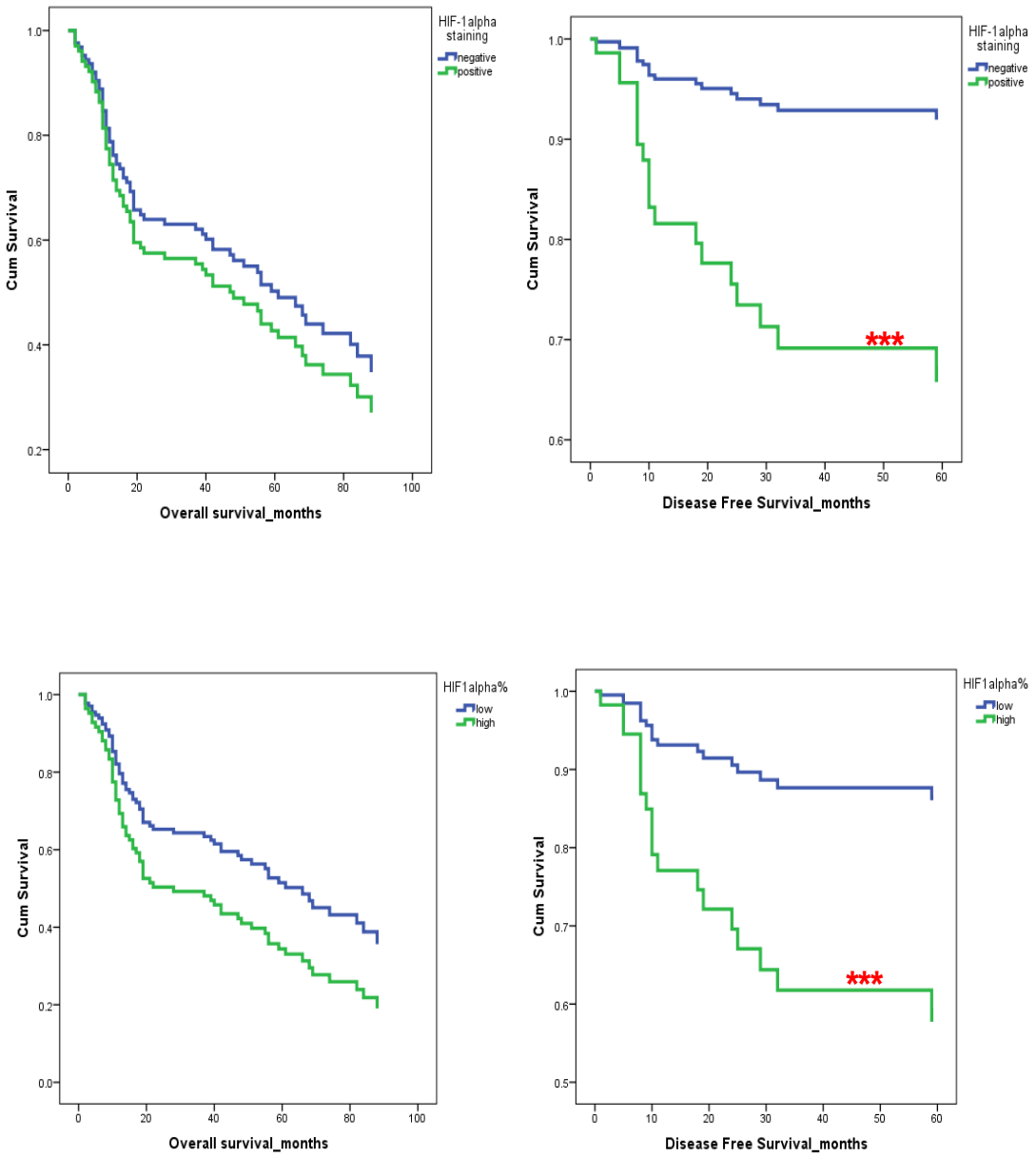
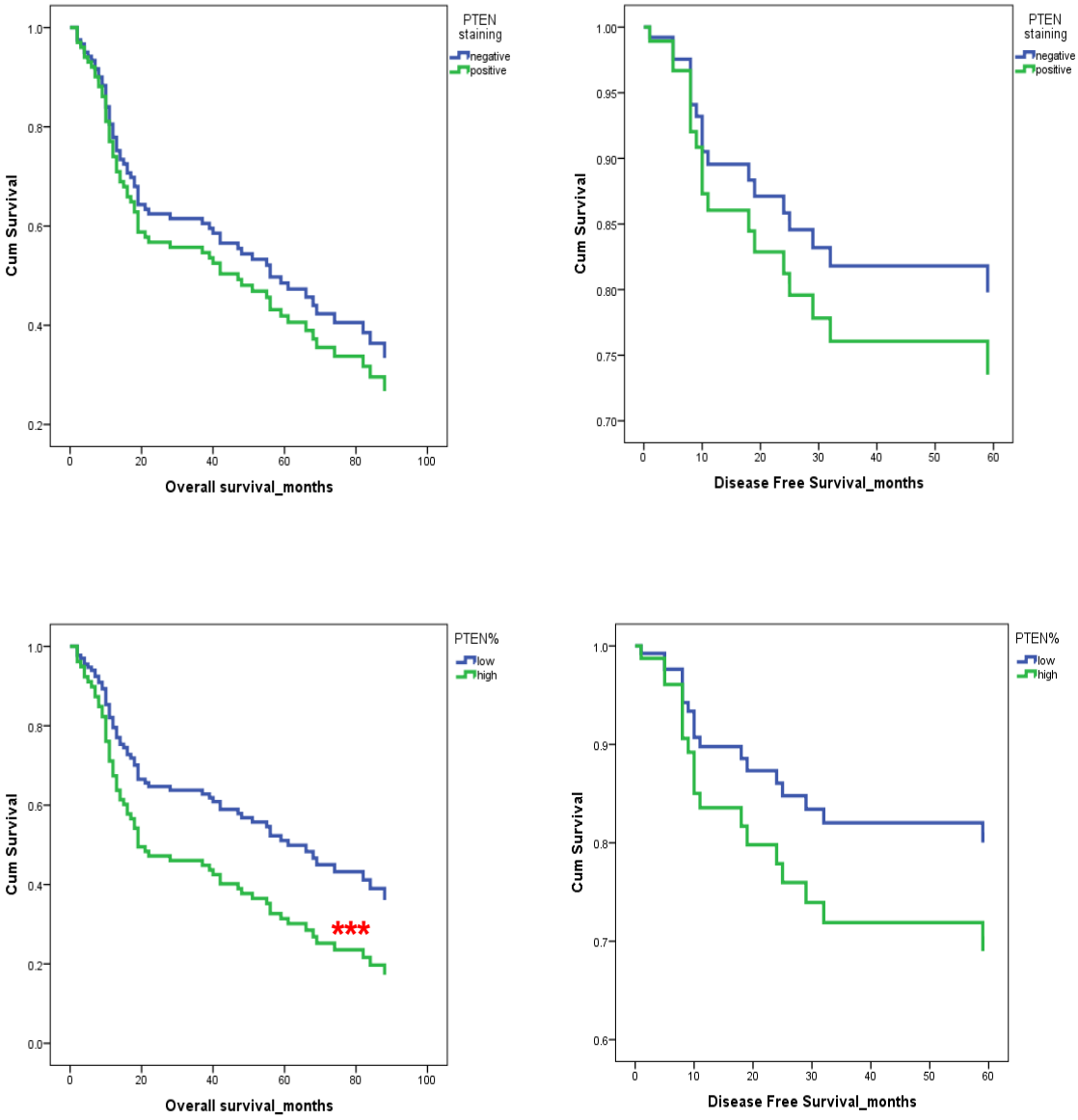


Figure 6.9 Survival analysis in relation to ERCC1 expression in HNSCC



*** Statistically significant *P* value

Figure 6.10 Survival analysis in relation to HIF-1α expression in HNSCC



*** Statistically significant *P* value

Figure 6.11 Survival analysis in relation to PTEN expression in HNSCC

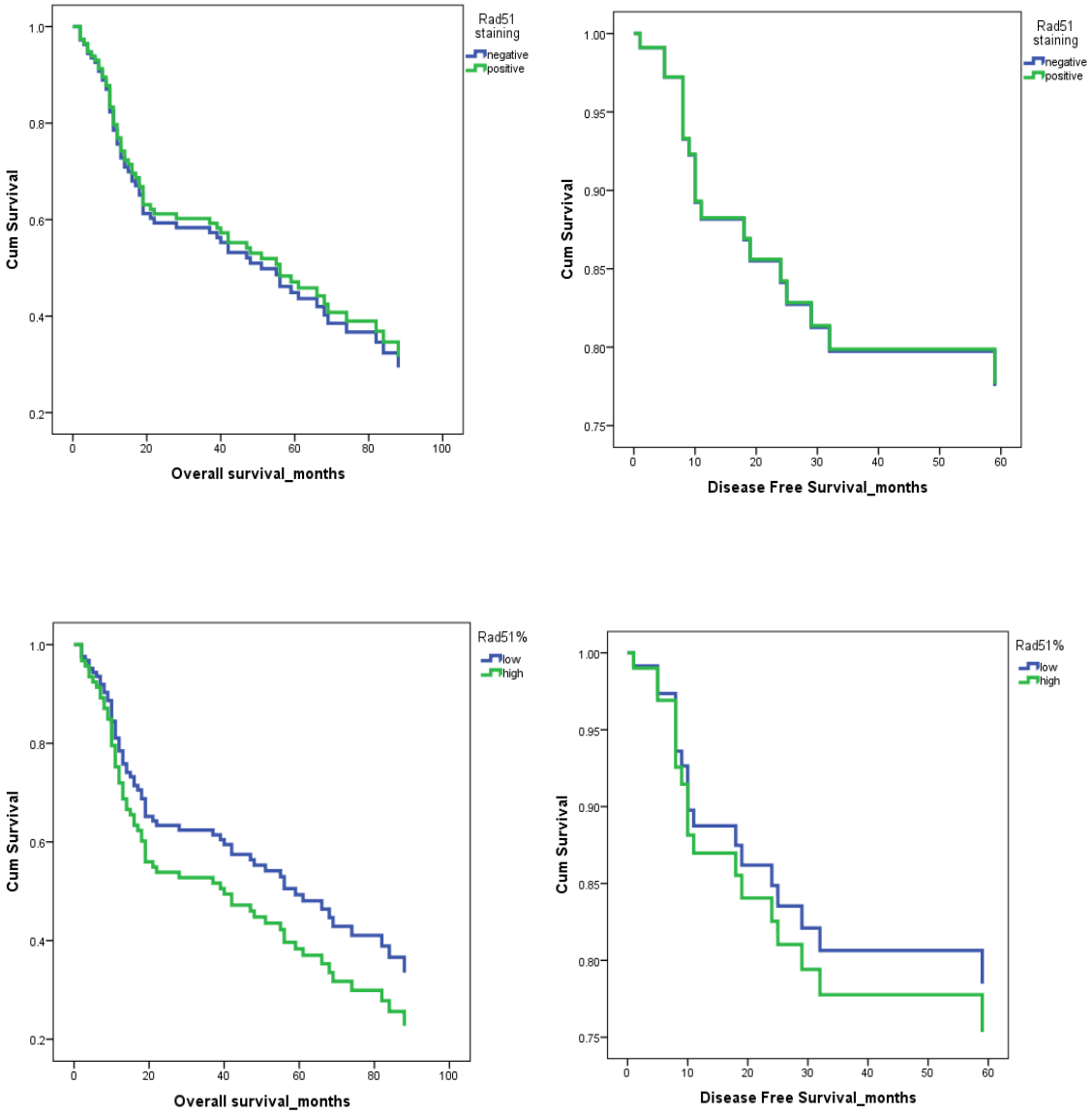


Figure 6.12 Survival analysis in relation to Rad51 expression in HNSCC

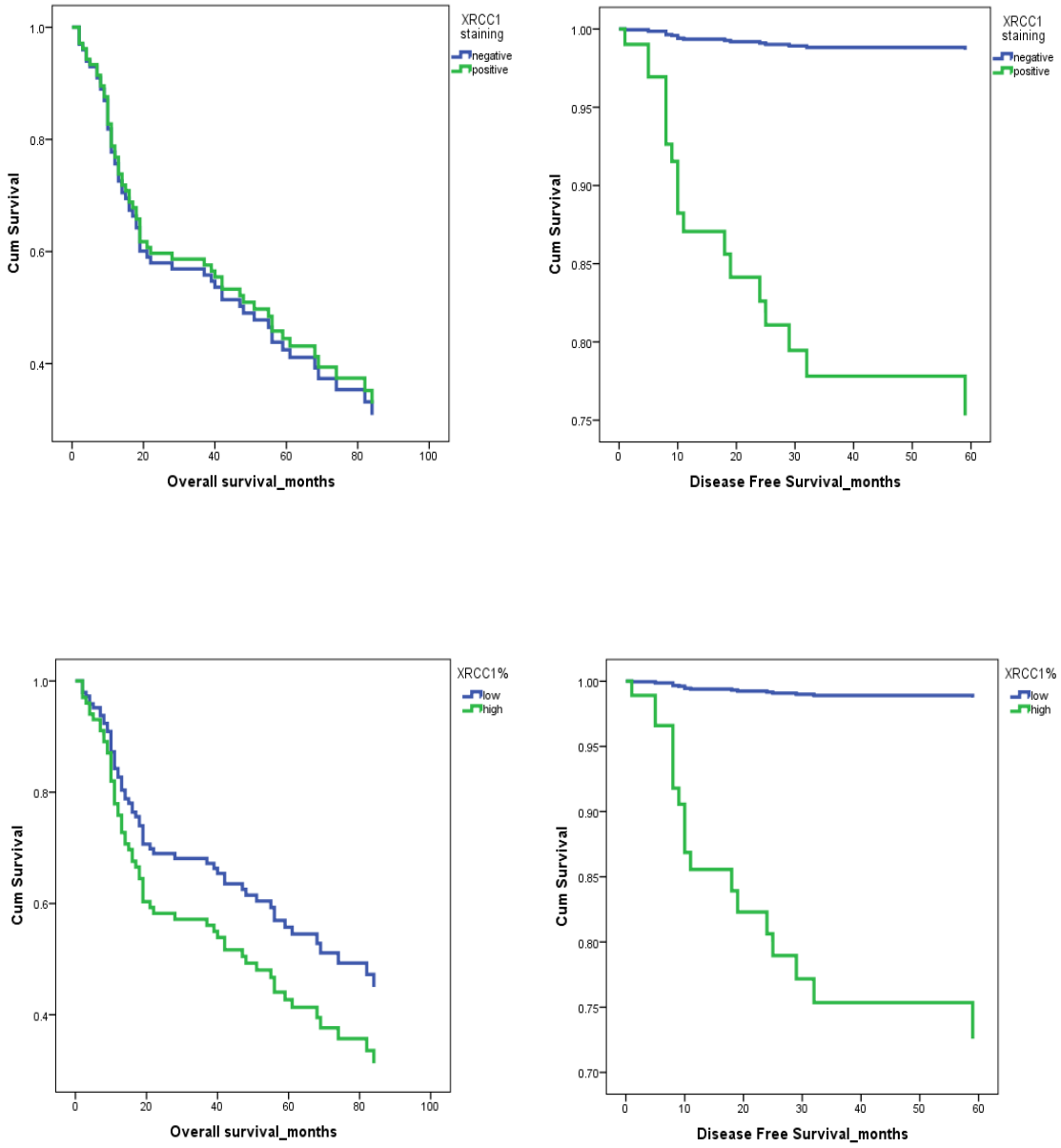


Figure 6.13 Survival analysis in relation to XRCC1 expression in HNSCC

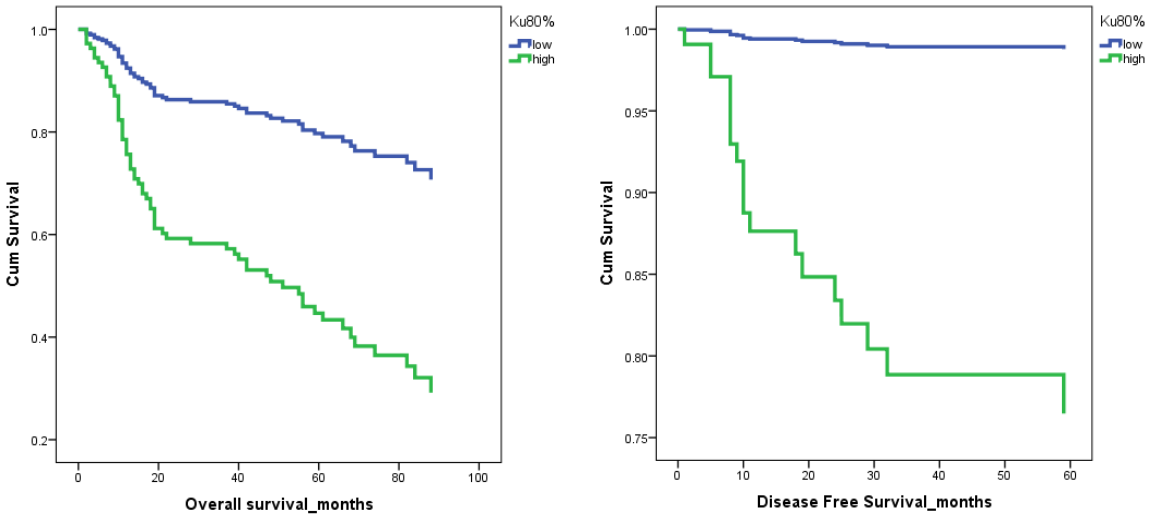


Figure 6.14 Survival analysis in relation to Ku80 expression in HNSCC

6.4 Association analysis

The association between protein expression and response to treatment (all types, excluding prior radiotherapy, 136 cases) or surgery followed by radiotherapy (69 cases)) was tested using the Chi-square/Fisher exact test. Logistic regression analysis was carried out to identify the significant predictors of response. The main purpose of this analysis was to determine whether the expression of proteins involved in the DNA damage repair and tumour hypoxia could predict the outcome of treatment, in particular to radiotherapy. Disease free survival was used as an indicator of the treatment outcome. Response to treatment was reduced to a binomial score on the basis of survival distribution. A scatter plot of survival of the 136 cases is shown in Figure 6.15 and it can be seen that survival naturally clusters into short and long term survivor with a natural division at 32-36 months. Patients who survived more than three years without developing any recurrence were considered good responders to treatment. Conversely patients who developed recurrence or died of carcinoma in less than three years were considered bad responders to treatment. In this analysis the 14 post-radiotherapy cases were excluded.

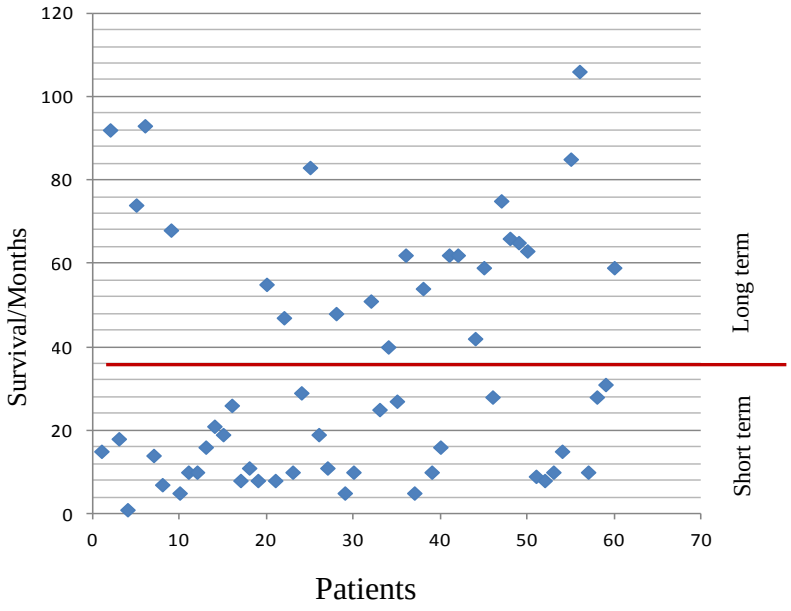


Figure 6.15 Scatter plot of disease free survival of study cases

6.4.1 Association analysis in relation to treatment

The variables tested were gender, site of origin, tumour differentiation, TNM stage, lymph node metastasis, expression level of ERCC1, HIF-1α, Ku80, PTEN, Rad51 and XRCC1 (intensity and percentage). The *p* values are listed in Table 6.5.

- ERCC1 intensity, PTEN intensity, lymph node metastasis, TNM stage and site of origin, significantly associated with the response to treatment.
- 61.8% of ERCC1 negative carcinomas showed a better response to treatment. While only 41% of ERCC1 positive carcinomas were good responders to treatment (p=0.010).
- 71% of carcinomas which had a high percentage of PTEN positive cells were bad responders to treatment and 55% of low PTEN expressing tumours had good outcome to treatment (p=0.016).
- 66% of carcinomas with lymph node metastasis at diagnosis had poor outcome to treatment while 58.3% of carcinomas without lymph node metastasis had a better response to treatment (p=0.010).
- Stage 3 and stage 4 tumours had a higher percentage of bad responders (78.9% and 55% respectively) than stage 1 and stage 2 tumours (38.5% and 33.3% respectively) (p=0.022).

Variables with (P<0.10) were included in the logistic regression analysis to identify the significant predictors of response. PTEN low expression was significant predictor of good response (p =0.018). Lip vermillion as a site of origin was also associated with better response to treatment (p =0.025) (Table 6.5).

6.4.2 Association analysis in relation to surgery followed by radiotherapy treatment

The same analysis was performed for the 69 cases treated by surgery followed by radiotherapy only. The *p* values are listed in Table 6.5.

- ERCC1 intensity, HIF-1α intensity, percentage and gender significantly associated to the outcome.
- 73.3% of ERCC1 positive carcinomas had poor treatment outcome. While 65% of ERCC1 negative carcinomas responded well to treatment by surgery followed by radiotherapy (p=0.004).
- 71.9% of HIF-1α positive carcinomas and 85% carcinomas with high percentage of HIF-1α expressing cells didn't respond well to treatment by surgery followed by radiotherapy (p=0.046 and p=0.005 respectively).
- 92.3% of females responded badly to treatment with surgery followed by radiotherapy (p=0.009).

Variables with (P<0.10) were included in the logistic regression analysis to identify the significant predictors of response, none of the variables showed significant association with response to treatment by surgery and radiotherapy (Table 6.5)

Table 6.5 Left: Chi-square/Exact test *p* values. Right: logistic regression results of response to treatment association analysis

Response to all types of treatments

Variable	χ 2 *p* value
Gender	0.908
Site	**0.048**
Differentiation	0.685
TNM stage	**0.022**
L.N metastasis	**0.010**
ERCC1	
Intensity	**0.043**
Percentage	1.00
HIF-1α	
Intensity	0.373
Percentage	0.102
Ku80	
Intensity	*
Percentage	0.666
PTEN	
Intensity	0.062
Percentage	**0.016**
Rad51	
Intensity	0.296
Percentage	0.146
XRCC1	
Intensity	0.597
Percentage	0.399

Variable	*P* value	OR	95% CI
Site			
Lateral tongue	0.06		
Buccal mucosa	0.532	0.60	0.12-2.9
Retr-palate	0.338	2.24	0.42-11.76
Floor of mouth	0.067	0.19	0.032-1.12
Alveolus	0.758	1.34	0.2-8.62
Lip	**0.025**	0.46	0.003-0.679
Tonsils	0.097	4.24	0.76-23.46
Pharynx	0.902	0.89	0.164-4.92
TNM stage			
Stage 1	0.349		
Stage 2	0.157	0.25	0.03-1.69
Stage 3	0.09	0.16	0.022-1.31
Stage 4	0.236	0.32	0.04-2.1
L.N metastasis	0.104	0.35	0.1-1.23
ERCC1 intensity	0.297	0.54	0.77-1.7
PTEN intensity	0.703	1.33	0.3-5.94
PTEN percentage	**0.018**	0.108	0.01-0.68

Response to all types of surgery+radiotherapy

Variable	χ 2 *p* value
Gender	**0.009**
Site	0.070
Differentiation	0.142
TNM stage	0.075
L.N metastasis	0.432
ERCC1	
Intensity	**0.004**
Percentage	1.00
HIF-1α	
Intensity	**0.046**
Percentage	**0.005**
Ku80	
Intensity	*
Percentage	0.513
PTEN	
Intensity	0.408
Percentage	0.304
Rad51	
Intensity	0.362
Percentage	0.458
XRCC1	
Intensity	*
Percentage	0.354

Variable	P value	OR	95% CI
Gender	0.075	9.77	0.74-120
Site			
Lateral tongue	0.945		
Buccal mucosa	0.999	0.00	0.000
Retr-palate	1.0	2618	0.000
Floor of mouth	0.839	0.79	0.83-7.57
Alveolus	0.664	0.5	0.027-9.39
Lip	0.999	0.00	0.000
Tonsils	0.282	3.86	0.329-45.29
Pharynx	0.447	2.72	0.206-36.02
TNM stage			
Stage 1	0.442		
Stage 2	0.773	1.75	0.038-79.9
Stage 3	0.365	0.17	0.004-7.71
Stage 4	0.825	1.4	0.68-29-15
ERCC1 intensity	0.085	0.16	0.022-1.27
HIF-1α intensity	0.624	0.61	0.09-4.24
HIF-1α percentage	0.596	0.54	0.5-5.1

*calculation of *p* value was not possible because all cases were immunopositive.

6.4.3 HPV status in tonsil and pharyngeal carcinomas

6.4.3.1 P16 immunostaining

20 tonsil and 15 pharyngeal cases were selected for immunostaining by p16 using TMA sections on the basis of site of origin. Out of the 35 carcinomas only 8 cases were p16 positive (Figure 6.16A) using criteria of strong and diffuse staining in 95% of cells. Whole sections of these eight cases were subjected to DNA ISH for detection of HPV DNA. Six carcinomas were HPV positive (Figure 6.16B).

6.4.3.2 HPV status and survival

Survival analysis was performed for the 35 tonsil and pharyngeal carcinomas in relation to their HPV status using Cox Regression analysis. HPV negative carcinomas were more likely to kill the patient than HPV positive carcinomas (*P* value= 0.061, OR=6.8, 95% CI=0.92-51.4) (Figure 6.17). In relation to disease free survival, the number of cases in each category (HPV positive and negative) was not enough to perform the analysis.

6.4.3.3 HPV status and response to treatment

83.3% of HPV positive carcinomas were good responders to treatment and 58.8% of HPV negative carcinomas were bad responders. However, it was not statistically significant (*P* value= 0.15). Association analysis between response to treatment and expression of ERCC1, HIF-1α, Ku80, PTEN, Rad51 and XRCC1 protein were re performed after excluding the HPV positive carcinomas and the results were unchanged except for PTEN expression (staining intensity) which became significantly associated with response to treatment (*P* value= 0.04) and showed that 67% of PTEN positive carcinomas were bad responders.

6.4.3.4 HPV status and expression of ERCC1, HIF-1α, Ku80, PTEN, Rad51 and XRCC1

Association between expression of ERCC1, HIF-1α, Ku80, PTEN, Rad51 and XRCC1 protein and HPV positivity in tonsil and pharyngeal carcinomas was

analysed using Chi-square/Fisher exact test. No significant association was found between HPV positivity and expression of these biomarker proteins (*P* values= 1.00 for all tested proteins).

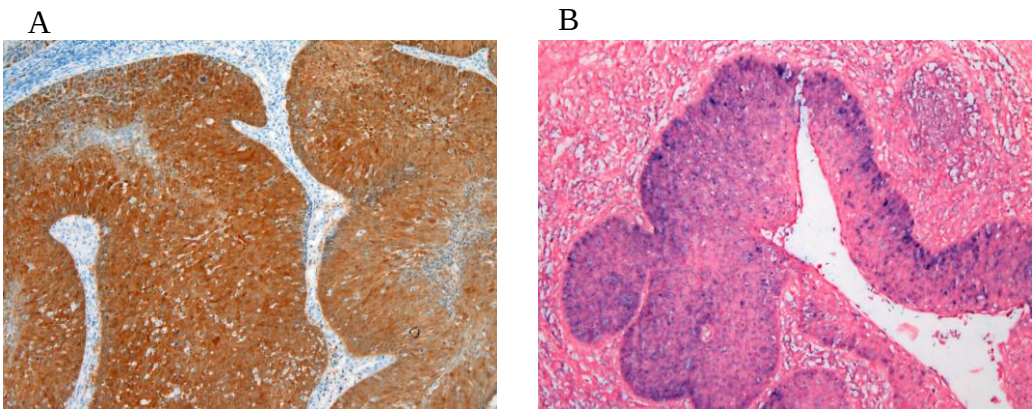


Figure 6.16 P16 immunostaining and HPV ISH in tonsil carcinomas

Photomicrographs showing representative immunostaining for p16 (A) and HPV in situ hybridization (B) in tonsil carcinomas. Original magnification10x objective, direct to 5M pixel camera.

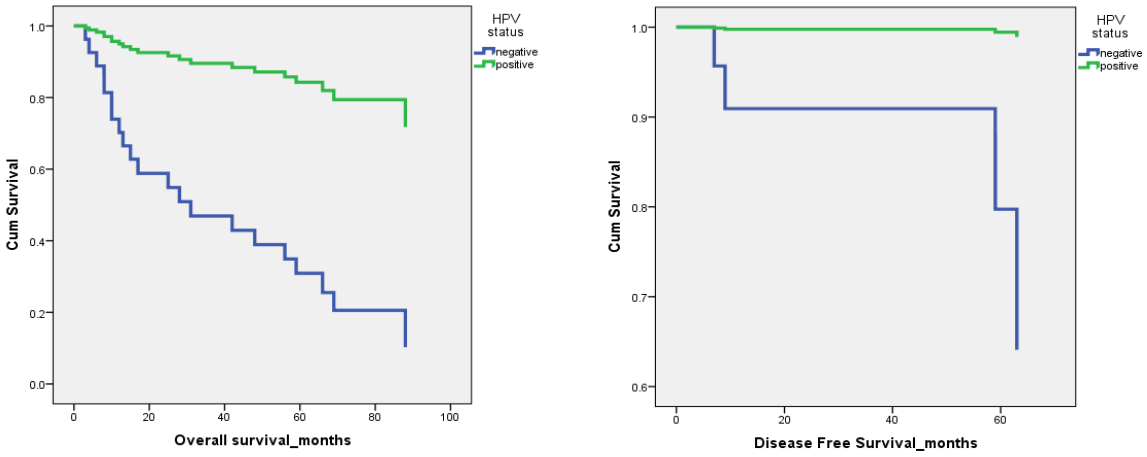


Figure 6.17 Survival analysis in relation to HPV status

In summary, in this chapter, the expression of proteins involved in DNA damage repair and tumour hypoxia was investigated. Furthermore, this expression level was correlated with overall survival, disease free survival and treatment outcome in the study cases. The main findings in this chapter are:

- Lack of, or low, expression of HIF-1α was associated with better disease free survival and was significant predictor of good response to treatment by surgery followed by radiotherapy.
- ERCC1 expression was associated with bad response to all treatment types.
- Low expression of PTEN was a predictor of better overall survival and a significant predictor of good response to treatment.
- Ku80, Rad51 and XRCC1 expression were not associated with response to treatment by any modality or combination.
- Metastasis to lymph node was a significant factor in relation to overall survival and disease free survival. Cases without lymph node metastasis showed better survival than cases with lymph node metastasis at time of diagnosis. Furthermore, lack of metastasis to lymph node was a predictor of good response to treatment.
- TNM stage 3 and 4 were associated with poor outcome to treatment.
- Tumour differentiation had no effect on survival or response to treatment
- In multivariate analysis, only low percentage of PTEN expressing cells and lip as a site of origin were predictors of good response to treatment.
- In tonsil and pharyngeal carcinomas, patient with HPV positive oropharyngeal carcinomas had better overall 3 years survival rates and better response to treatment than patients with HPV negative tumours.

- Expression of ERCC1, HIF-1α, Ku80, PTEN, Rad51 and XRCC1 protein was not associated with HPV status of oropharyngeal carcinomas.

7 Discussion

Head and neck cancer is the 6th most common cancer with high morbidity and mortality rates worldwide. Despite advances in the treatment of head and neck cancer over the last several years, its survival rates remain poor so there is an urgent need for specific and effective treatments. Surgery alone or combined with radiotherapy and/or chemotherapy is currently the main type of treatment for head and neck cancer. Other treatment modalities based on better understanding of the molecular nature of these tumours have emerged in the last few years but have had limited success.

TRAIL and Smac mimetics have shown anti-cancer activity in several types of cancer cell lines as well as in mouse tumour models. TRAIL based therapeutics have entered phase I and Phase II clinical trials for the treatment of advanced solid and haematological cancers. Little is known about the effect of TRAIL and Smac mimetics on head and neck cancer. In this study we investigated the cytotoxic effect of TRAIL and Smac mimetics on a panel of HNSCC cell lines. The underlying molecular mechanisms of TRAIL and Smac mimetics induced cell death were also investigated. Furthermore, this study sought to identify the biomarkers which could predict the response of HNSCC cell lines to TRAIL and Smac mimetic treatment.

The main pitfall of treatment with chemo-radiotherapy is that a high percentage of patients develop resistance to radiotherapy and or chemotherapy, which results in poor treatment outcome as well as undesirable side effects. For such patients, new and alternative therapeutic agents are necessary. Therefore, identifying those patients who will not benefit from a certain treatment modality can spare patients suffering as well as cost.

In this study we investigated the expression of a number of genes involved in the DNA damage repair process in primary carcinoma tissues of 150 HNSCC patients. Association between protein expression profile and survival as well as the treatment outcome of these patients was analysed.

7.1 TRAIL and Smac59 killed HNSCC cell lines effectively

Recombinant IZ-TRAIL has been shown to induce apoptosis in leukaemia, multiple myeloma, neuroblastoma, lung, liver, colon, breast, prostate, pancreas, kidney and thyroid cancer cell lines (Newsom-Davis et al., 2009). IZ-TRAIL was not toxic to primary human hepatocytes (Ganten et al., 2006). These findings promoted TRAIL as a promising anticancer drug and it entered into clinical trials. Smac mimetics have been shown to induce apoptosis as a single agent in glioblastoma (Li et al., 2004), leukaemia (Sun et al., 2007) as well as breast cancer cell lines (Lu et al., 2008). Smac mimetics as a single agent induced cell death in NSCLC cell lines as well as tumour regression in lung xenograft model. Furthermore, Smac mimetics were safe to normal cells (Petersen et al., 2007). The effect of TRAIL and Smac mimetics on HNSCC cell lines or normal keratinocytes has been tested and the results were shown in section 3.1.

In this study we have shown that IZ-TRAIL and Smac59 effectively kill HNSCC cell lines as a single agent at low concentrations. HNSCC cell lines were found to be sensitive either to TRAIL or Smac59; out of the nine HNSCC cell lines tested, five were sensitive to IZ-TRAIL and three were resistant indicating that HNSCC cells are more sensitive to TRAIL than most other primary cancer cell lines which are found to be TRAIL resistant. This may be due to an inherent susceptibility of HNSCC cell lines to TRAIL.

Only three out of the nine HNSCC cell lines tested were Smac59 sensitive, however their sensitivity was remarkable. Smac59 was able to induce cell death at fractions of nanomolar concentration. Smac59 is a bivalent Smac mimetic and is known for its high activity, however it was not able to induce cell death as a single agent in our resistant cells even at high concentrations up to 1μM (data not shown). Similarly, Sun et al. found that Smac164 (same as Smac59) could not induce cell death as a single agent in 1483, 1640, JHU HNSCC cell lines (Sun et al., 2011). However, using both TRAIL and Smac59 was able to kill all tested cell lines at low concentrations. These data suggested that TRAIL and Smac59 could be potential therapeutic agents for treating HNSCC.

The pattern of sensitivity observed in our HNSCC cell lines provided a good model for studying the cellular mechanisms that determine the differences between sensitive and resistant cells as well as identifying biomarkers that might allow the prediction of response of different cells to TRAIL and Smac mimetics prior to treatment.

7.1.1 Smac59 sensitised TRAIL resistant cell lines to TRAIL

In this study we have used Smac59 at non toxic concentrations to sensitise TRAIL resistant cell lines (HSC3, HSC3M3 and HN5) and although Smac59 was able to sensitise HSC3, HSC3M3 cells to TRAIL, the resultant effect was significantly less than the effect of Smac59 alone when used at toxic concentration in these cell lines. Furthermore, Smac59 was not able to sensitise HN5 cells to TRAIL suggesting that this sensitisation effect may be cell type specific.

Smac mimetics have been used to sensitise several cancer cell lines to TRAIL (Fandy et al., 2008; Li et al., 2004; Sun et al., 2011). In these studies Smac mimetics were not able to induce cell death alone but in our study Smac59 was highly toxic to the sensitive cells suggesting that using Smac59 as a single agent is more effective than being used as a sensitiser to TRAIL. Our findings therefore suggest that single treatment with either Smac59 or TRAIL is sufficient in killing different HNSCC cell lines. However, to select specific cases for treatment with suitable drug, understanding the molecular mechanisms that determine the response to this targeted therapeutics is important.

7.1.2 TRAIL but not Smac59 was toxic to normal human keratinocytes

TRAIL was shown to be effective in killing most of the cancer cell lines tested in this study but it was also toxic to normal oral keratinocytes and a non-transformed, immortalised epidermal keratinocytes, which may limit its use in patients. On the other hand Smac59 had no effect on the viability of normal oral keratinocytes suggesting it as a safe therapeutic in patients. A previous study by Ganten et al. showed that primary hepatocytes were sensitive to TRAIL in early

days of their culture but they develop resistance to TRAIL with time during culture in vitro (Ganten et al., 2006) Furthermore, the toxicities observed for some forms of recombinant TRAIL were suggested to represent either cell culture artefacts or are related to the specific recombinant protein used (Newsom-Davis et al., 2009). In this study it was not possible to keep primary normal cells in culture for long enough to test if they will develop resistant to TRAIL, however the HaCat cells were sensitive to TRAIL even during long period of culture. IZ-TRAIL used in this study was shown to be safe in normal hepatocytes (Ganten et al., 2006). In vivo studies are necessary to further investigate the cytotoxic effect of TRAIL and Smac59 on normal and non-transformed tissues including oral keratinocytes.

7.1.3 Protein biomarkers could predict response of HNSCC to TRAIL and Smac59

The observed pattern of sensitivity of HNSCC cell lines triggered further investigation into the underlying genetic causes, particularly the expression level of proteins that play a role in pathways involved in TRAIL and Smac mimetics-induced apoptosis. IAPs (XIAP, cIAP-1 and cIAP-2), caspases (-3 and -8), Bcl-2, Mcl-1, Smac/DIABLO, cFLIP and EGFR expression levels were investigated using western blot analysis. TNF-α level was investigated using ELISA. TRAIL death receptors (DR4 and DR5) levels were investigated using flow cytometry. Additionally, the mRNA and microRNA expression profile of the cells grouped based on their resistance/sensitivity to these drugs was carried out and the followings are proteins which showed differential expression in TRAIL/Smac59 sensitive/ resistant cell lines.

7.1.3.1 Inhibitor of apoptosis proteins (IAPs)

In this study, XIAP level was higher in Smac59 sensitive (TRAIL resistant) cell lines than in Smac59 resistant (TRAIL sensitive) cell lines (Figure 4.1and Figure 4.2) while cIAP-2 expression was higher in Smac59 resistant (TRAIL sensitive) cell lines (Figure 4.3). XIAP is known to be overexpressed in many cancer cell lines and tumours compared to normal cells (Dai et al., 2009a). The

normal human keratinocytes (NHK) used in this study were found to express high levels of XIAP, however HaCat cells had a very low XIAP. In general, all HNSCC cells expressed high levels of XIAP which was in accordance with previous findings in other tissues for example in chronic lymphocytic leukaemia cells, XIAP expression level was significantly higher than normal cells (Frenzel et al., 2011). Why our normal cells expressed high level of XIAP could be due to temporary upregulation of XIAP expression as a protective mechanism in early days of culture and that could be changed overtime. It was not possible for us to test this hypothesis because of difficulties in maintain NHK in culture for long periods as mentioned above.

High levels of XIAP are believed to result in apoptosis resistance of cancer cells to a wide variety of therapeutic agents. In melanoma cells, TRAIL resistance was correlated with the ratio of XIAP and caspase-3 levels of the cells (Thayaparasingham et al., 2009), and XIAP expression has been proposed as an important adverse biomarker for chemoresistance in cancer patients (Kashkar, 2010). All the cell lines tested here expressed high levels of XIAP yet showed high sensitivity to either TRAIL or Smac59 suggesting either that these drugs were successful in overcoming inhibitory effect of XIAP in the sensitive cells or that XIAP is not involved in TRAIL or Smac59 resistance in HNSCC cell lines. Seeger et al. found that elevated XIAP expression alone cannot serve as a predictive marker of chemoresistance (Seeger et al., 2010).

cIAP-2 was less expressed in Smac59 sensitive (TRAIL resistant) cell lines than Smac59 resistant (TRAIL sensitive) cell lines indicating that maintaining low levels of cIAP-2 may be essential for Smac mimetic sensitivity. Knowing that some cancer cell lines evade Smac mimetic-induced apoptosis via upregulation of cIAP-2 expression (Petersen et al., 2010) is supporting this conclusion.

7.1.3.2 Smac/DIABLO

In this study we have shown that Smac/DIABLO expression level was higher in Smac59 sensitive (TRAIL resistant) cell lines, whilst Smac resistant cells

(TRAIL sensitive) had moderate levels of Smac/DIABLO (Figure 4.4). Expression of Smac/DIABLO has been shown to be important in determining the response of HNSCC cell lines to different chemotherapeutics that function through mitochondrial pathway activation (Sun et al., 2011). Smac/DIABLO promotes apoptosis through binding to XIAP in order to relieve its inhibitory effect on caspases. In Smac59 sensitive (TRAIL resistant) cell lines, XIAP and Smac/DIABLO levels were higher than in Smac59 resistant (TRAIL sensitive) cell lines which may indicate that these cell lines induce apoptosis mainly through the intrinsic apoptotic pathway.

7.1.3.3 Bcl-2 family members (Bcl-2 and Mcl-1)

Bcl-2 expression was detected in four out of six Smac59 resistant (TRAIL sensitive) cell lines and was undetectable in two out three Smac59 sensitive (TRAIL resistant) cell lines (Figure 4.5). Mcl-1 expression was observed in all tested cell lines but its level was higher in Smac59 sensitive (TRAIL resistant) cell lines (Figure 4.6). Anti-apoptotic activity of Bcl-2 and Mcl-1 is mediated through inhibition of mitochondrial pathway activation and release of cytochrome c, so overexpression of these proteins could be responsible for resistance of cell lines to drugs that work through the mitochondrial pathway. Whether Bcl-2 and Mcl-1 overexpression could be used as a biomarker for TRAIL and Smac mimetic sensitivity is controversial. Some studies have shown that overexpression of Bcl-2 protein mediates resistance to TRAIL induced apoptosis in neuroblastoma, glioblastoma and breast carcinoma cell lines (Fulda et al., 2002). Similarly, inhibition of Mcl-1 upregulation was shown to be able to sensitise neuroblastoma cells to TRAIL (Ammann et al., 2009) and that sensitisation of transformed and non transformed cells to TRAIL-induced apoptosis was associated with reduction in Mcl-1 expression levels (Martin-Perez et al., 2012). On the other hand, no difference in the expression level of Bcl-2 and Mcl-1 was found between primary colon carcinoma cell line SW480 (TRAIL sensitive) and the metastatic cell line SW20 (TRAIL resistance) (Ndozangue-Touriguine et al., 2008).

7.1.3.4 Caspases (caspase-8 and caspase-3)

Initiator caspase-8 activation is the first step in response to extrinsic apoptosis pathway activation; according to the level of caspase-8, cells decide whether or not to activate the intrinsic pathway to execute apoptosis (Scaffidi et al., 1998). The HNSCC cell lines tested here had clear differences in caspase-8 expression levels between Smac59 sensitive (TRAIL resistant) cell lines and Smac59 resistant (TRAIL sensitive) cell lines (Figure 4.7). Caspase-8 level was clearly lower in Smac59 sensitive (TRAIL resistant) cells, which suggests this as one of the mechanism responsible for resistance to TRAIL-induced apoptosis. Caspase-3 was expressed in all HNSCC cell lines and showed no correlation with sensitivity or resistance to these drugs.

7.1.3.5 Cellular FLICE-inhibitory protein (c-FLIP)

c-FLIP was expressed in almost all HNSCC cell lines as well as normal human keratinocytes and HaCat cells. Smac59 sensitive (TRAIL resistant) cell lines had lower levels of c-FLIP than Smac59 resistant (TRAIL sensitive) cell lines (Figure 4.9). The level of c-FLIP was directly correlated with the caspase-8 level, which suggests that cells produce c-FLIP at such levels to antagonize caspase-8 activation and apoptosis induction. Interestingly, normal human keratinocytes and HaCat cells had high levels of c-FLIP despite having low caspase-8 expression. c-FLIP inhibits caspase-8 auto-proteolysis at DISC which results in apoptosis inhibition (Krueger et al., 2001). Level of c-FLIP protein has been shown to influence the sensitivity of cell lines to TRAIL induced apoptosis and that sensitisation of transformed and non-transformed cells to TRAIL-induced apoptosis was related to reduction in the levels of FLIP (Martin-Perez et al., 2012).

7.1.3.6 Epidermal growth factor receptor (EGFR)

EGFR expression pattern in HNSCC cell lines was interesting as all Smac59 resistant (TRAIL sensitive) cell lines had low or undetectable level of EGFR in comparison to Smac59 sensitive (TRAIL resistant) cell lines which had high EGFR levels (Figure 4.10). EGFR activation is responsible for many pro-survival

mechanisms including cell growth, angiogenesis, inhibition of apoptosis, cell adhesion, cell motility and invasion (Lui and Grandis, 2002). Whether and how EGFR expression is involved in determining sensitivity or resistance of TRAIL and Smac59 is not clear. One possibility is that high EGFR expression may affect the binding of TRAIL to its receptors on cell membrane, or that activation of EGFR downstream pathways might be involved in blocking TRAIL induced apoptosis pathway leading to apoptosis inhibition, as seen in TRAIL resistant cells.

7.1.3.7 Tumour necrosis factor-alpha (TNF-α)

One of the mechanisms by which Smac mimetics induce apoptosis is through the activation of the TNF-α pathway (Dai et al., 2009a). Therefore the level of secreted TNF-α in HNSCC cell lines was measured using ELISA. Additionally TNF-α mRNA level was tested using gene expression profiling. Interestingly, Smac59 sensitive (TRAIL resistant) cells secreted higher levels of TNF-α than Smac59 resistant (TRAIL sensitive) cell lines (Figure 4.11). TNF-α mRNA level was also higher in Smac59 sensitive (TRAIL resistant) cell line (Figure 4.14). These findings suggested that TNF-α level strongly correlates with Smac59 sensitivity. This is in agreement with previous finding in other cancer cell lines, in which autocrine TNF-α secretion was a strong predictor of sensitivity to JP1201 Smac mimetics in non-small cell lung carcinoma cell lines (Greer et al., 2011).

7.1.3.8 TRAIL receptors

TRAIL death receptors DR4 and DR5 expression on HNSCC cell membrane was measured using flow cytometric analysis and gene expression profiling. Results from flow cytometry showed that DR4 and DR5 expressed in all cell lines tested regardless of the type of response to TRAIL (Figure 4.12). The results of gene expression profiling showed that Smac59 sensitive (TRAIL resistant) cell lines expressed higher mRNA levels for DR5 than Smac59 resistant (TRAIL sensitive) cell lines (Figure 4.14). Level of TRAIL death receptors expression and its importance for TRAIL-induced apoptosis is different according to cell lines tested. Martin-Perez et al. have shown that sensitisation of transformed cell lines to

TRAIL was in part due to upregulation of DR5 (Martin-Perez et al., 2012). However other studies have shown that sensitisation to TRAIL in malignant human glioma cells was not associated with overexpression of TRAIL receptors DR4 and DR5 (Jane et al., 2011).

Previous reports attributed resistance to TRAIL to overexpression of TRAIL decoy receptors DcR1 and DcR2 by competing with DR4 and DR5 for TRAIL binding (Newsom-Davis et al., 2009). In this study, gene expression profiling showed that DcR1 and DcR2 expression was not different between TRAIL sensitive and TRAIL resistant cell lines.

7.1.4 miRNA biomarkers could predict response of HNSCC to TRAIL and Smac59

Analysis of miRNA expression profile in HNSCC cell lines revealed a significant difference in the expression of a number of miRNAs between Smac59 sensitive (TRAIL resistant) cell lines and Smac59 resistant (TRAIL sensitive) cell lines (Figure 4.15). These could be potential biomarkers for predicting sensitivity to TRAIL or Smac59. 15 miRNA were upregulated and 7 miRNA were downregulated in Smac59 resistant (TRAIL sensitive) cell lines. The validation of these biomarkers needs further investigations. However, miR-9 as the most upregulated miRNA in Smac59 resistant (TRAIL sensitive) cells is an important candidate. NF-κB is one of the validated targets of miR-9 and NF-κB is a transcription factor for many proteins including TNF-α (Pahl, 1999).

We have shown that TNF-α level is higher in Smac59 sensitive (TRAIL resistant) cell lines (Figure 4.11) which indicates that NF-κB transcriptional activity may be higher. This assumption is consistent with previous findings which showed that blocking NF-κB activation reduced TNF-α production and protected cells to Smac mimetic induced cell death (Ahn et al., 2007) and that activated NF-κB enhanced the ultraviolet B radiation induced apoptosis via NF-κB dependant increased secretion of TNF-α (Barisic et al., 2008). It is tempting to speculate that the low level of TNF-α in Smac59 resistant (TRAIL sensitive) cell lines is due to

negative regulation through miR-9-mediated inhibition of NF-κB activity in these cell lines. However, these observations are preliminary and further investigations are in progress to validate these findings.

7.1.5 Smac59 increased the levels of secreted TNF-α in sensitive cell lines and TNF-α sensitised resistant cells to Smac59

Since the endogenous level of TNF-α correlated with Smac59 sensitivity and since one mechanism of Smac mimetics induced apoptosis is to induce autocrine TNF-α production (Lu et al., 2007), the effect of Smac59 and TRAIL on the level of TNF-α in HNSCC cell lines was tested. Smac59 was found to effectively increase the level of secreted TNF-α in the sensitive cell lines but not in the resistant cell lines (Figure 5.1), which suggested that Smac59 induced apoptosis in these cell lines is probably mediated by activation of the TNF-α pathway. To further investigate the role of TNF-α in Smac59 induced apoptosis in HNSCC cell lines, rhTNF-α was added to the cells alone or in combination with TRAIL or Smac59. Furthermore, TNF-α was inhibited by both blocking and neutralising antibody and its effect on cell sensitivity to Smac59 was measured.

Adding rh-TNF-α to Smac59 sensitive cell lines did not induce a similar level of apoptosis to that obtained with Smac59. Consistent with this, inhibiting TNF-α could not protect the sensitive cell lines from Smac59 induced apoptosis. Our findings are in agreement with Petersen and co-workers who found that adding Smac mimetics to sensitive non-small cell lung carcinoma cell lines activated TNF-α secretion whereas this effect was not observed in resistant cells. But by contrast to our results, using TNF-α neutralizing antibodies protected their cells from Smac mimetics induced apoptosis (Petersen et al., 2007). In their study 1-2 µg/ml of neutralizing antibody was used compared to 10µg/ml in our study. Therefore, it is unlikely that our finding is due to insufficient amount of neutralizing antibody used. We also used blocking antibody (20µg/ml) to inhibit TNF-α activity and the results were the same. Taken together, this part of the study suggests that in HNSCC cell lines, Smac59 utilizes other mechanisms in addition to stimulating autocrine

secretion of TNF-α. A previous study by Wagner and co-workers found that Smac mimetics sensitisation of glioblastoma cells was not associated with TNF-α production and that use of TNF-α blocking antibody could not inhibit this sensitisation (Wagner et al., 2012).

Interestingly, combining TNF-α with Smac59 sensitised Smac59 resistant cells suggesting that lack of TNF-α in this cell line may be the reason of their Smac59 resistance. This finding is in agreement with Li et al. studies which showed that combining TNF-α and Smac mimetic (compound 3) successfully induced apoptosis in HeLa cells which were resistant to either TNF-α or Compound 3 alone (Li et al., 2004). The exact mechanism by which TNF-α sensitised UMSCC11B to Smac59, and how TNF-α and NF-κB are involved in this mechanism needs further investigation.

In conclusion, the results obtained in our study suggest that TNF-α could be a marker of Smac59 sensitivity and that TNF-α could be used to sensitise Smac59 resistant cells to Smac59 induced apoptosis. However, the exact mechanism by which Smac59 utilizes the TNF-α pathway is not clearly understood. TNF-α alone was unable to induce cell death at levels that were achieved with Smac59. Furthermore, blocking TNF-α did not inhibit Smac59 induced apoptosis. Collectively, these results suggest that TNF-α is important for Smac59 induced apoptosis but that it was not the only factor involved in this process.

7.1.6 Bcl2 overexpression induced TRAIL resistance in TRAIL sensitive cells but could not protect Smac59 sensitive cells against Smac59-induced apoptosis

The pattern of Bcl-2 expression in HNSCC cell panel tested showed that most of Smac59 resistant (TRAIL sensitive) cells had high Bcl-2 levels while most of the Smac59 sensitive (TRAIL resistant) cells had undetectable levels. To further investigate the role of Bcl-2, overexpression experiments were performed. Treatment of a Bcl-2 overexpressing mixed population of HSC3M3 cell line (Smc59 sensitive) with Smac59 had a similar effect as parental HSC3M3 cells,

suggesting that Smac59 induced apoptosis may be independent or downstream of Bcl-2. To further understand the role Bcl-2 in Smac59 induced apoptosis, Bcl-2 knockdown in Smac59 resistant cell lines needs to be performed.

Conversely, Bcl-2 overexpression in H357 (TRAIL sensitive) cells mediated significant resistance to TRAIL treatment compared to parent cells suggesting, that in this cell line TRAIL induced apoptosis is at least partly regulated by Bcl-2 expression and that Bcl-2 overexpression could protect cells against TRAIL induced apoptosis. Our results are in agreement with the finding that overexpression of bcl-2 conferred protection against TRAIL in neuroblastoma, glioblastoma or breast cancer cell lines (Fulda et al., 2002).

As mentioned before most Smac59 resistant (TRAIL sensitive) cell lines expressed high levels of Bcl-2 protein though they were highly sensitive to TRAIL. This finding is not compatible with the overexpression experiments results. This could be explained as follows, H357 cells express undetectable or low levels of Bcl-2 and caspase-8 compared with other TRAIL sensitive cell lines. This suggests that TRAIL may induce apoptosis through different mechanisms in different cell lines; in H357 with low caspase-8, activation of the extrinsic pathway may not be sufficient to accomplish apoptosis and the involvement of mitochondrial pathway may be necessary and this may be why Bcl-2 overexpression protected H357 against TRAIL-induced cell death. Whilst in other cell lines with high levels of caspase-8, TRAIL activation of extrinsic pathway may be sufficient to accomplish apoptosis without the need for mitochondrial involvement and this could explain why Bcl-2 overexpression in these cells did not affect their sensitivity to TRAIL.

Therefore to understand the role of Bcl-2 in TRAIL induced apoptosis, it is important to identify whether the cells are type I or type II and which apoptotic pathway is activated to execute apoptosis.

7.1.7 TRAIL and Smac59 depleted XIAP and cIAP-1 in HNSCC

Understanding the underlying mechanisms of TRAIL and Smac59 induced apoptosis in HNSCC cell lines is crucial for future therapeutic application of these

drugs. One of the main mechanisms by which cancer cells evade apoptosis is the overexpression of inhibitor of apoptosis proteins and in particular XIAP. We investigated the role of XIAP and cIAP-1 in TRAIL and Smac59 induced apoptosis and the effect of TRAIL and Smac59 treatment on XIAP expression in HNSCC cell lines.

We have shown that all our HNSCC cells expressed relatively high levels of XIAP and cIAP-1 though all of them were highly sensitive to either TRAIL or Smac59. This suggested that in these cell lines high endogenous IAPs expression did not inhibit apoptosis induced by TRAIL or Smac59. Furthermore, we have shown that Smac59 sensitive (TRAIL resistant) cell lines had slightly higher XIAP levels than Smac59 resistant (TRAIL sensitive) cell lines.

Treatment with Smac59 resulted in the depletion of cIAP-1 in both Smac59 sensitive and resistant cell lines early after treatment (Figure 5.7), before caspase-3 activation and PARP cleavage (Figure 5.6). Smac mimetics are known to induce ubiquitination of cIAPs by direct binding which results in cIAPs degradation and consequently caspase activation (Chen and Huerta, 2009). In the HNSCC cell lines tested here, Smac59-mediated, cIAP-1 depletion did not result in caspase activation or apoptosis induction in Smac59 resistant cells indicating either that cIAP-1 depletion is not the determining factor of Smac59 induced apoptosis or that cIAP-1 upstream targets in these cell lines are blocked. Recently it has been shown that Smac mimetics induce rapid degradation of cIAP1 and cIAP2 and this requires the binding of TNF-α to its receptor (TRAF2) (Darding et al., 2011). It is unlikely that the same mechanism applies in HNSCC cell lines because Smac59 did not stimulate TNF-α secretion in Smac59 resistant (TRAIL sensitive) cells. TRAIL treatment induced cIAP-1 depletion in TRAIL sensitive cell line UMSCC11B at 8 hours which suggests that this depletion is a product of apoptosis and that cIAP-1 is not involved in apoptosis initiation by TRAIL in this cell line.

In this study, we have shown that both TRAIL and Smac59 induced XIAP depletion in their sensitive cells. The downregulation was observed at 8 hours after treatment and increased with time. Caspase-3 activation and PARP cleavage were

observed at 4 hours after treatment, which suggests that XIAP downregulation is a consequence rather than the cause of apoptosis. However, an important role of XIAP in TRAIL or Smac59 induced apoptosis cannot be ruled out as interactions could have taken place early during caspase-3 activation. Yang and co-workers showed that in HeLa cells, despite Smac/DIABLO promoting the auto-ubiquitination of XIAP, it did not result in XIAP degradation (Yang et al., 2004). In our cell lines, Smac59 could have induced ubiquitination of XIAP releasing its inhibitory effect on caspases and promoted apoptosis induction while XIAP degradation occurred late after apoptosis execution, such hypothesis need further investigation of the interaction between Smac59 and XIAP.

7.1.8 TRAIL and Smac59 mediated XIAP depletion was Caspase dependent, while Smac-59 mediated depletion of cIAP-1 was caspase independent.

Caspase inhibitor Z-VAD-fmk was used to investigate the effect of caspase inhibition on XIAP downregulation induced by TRAIL and Smac59 in sensitive cells. Pre-treatment with pan caspase inhibitor completely abolished both the TRAIL and Smac59 mediated downregulation of XIAP suggesting that XIAP depletion is caspase dependent. To determine which caspase was responsible for this effect, specific caspase inhibitors need to be used to clarify the exact mechanisms underlying TRAIL and Smac59 induced XIAP depletion. It has been shown that TRAIL induced depletion of XIAP and cIAP-1 required caspase activity and that caspase-8 knockdown stabilized both XIAP and cIAP-1, while caspase-9 knockdown prevented only XIAP, but not cIAP-1 degradation (Guicciardi et al., 2011).

In this study, results showed that Smac59 mediated cIAP-1 depletion was not affected by treatment with caspase inhibitor suggesting that Smac59 induced cIAP-1 degradation was independent of caspase activity in HNSCC cell lines. This is in agreement with previous studies that Smac mimetics induce cIAP-1

degradation via direct binding to their E3 motif which results in their ubiquitination (Chen and Huerta, 2009).

7.1.9 XIAP knockdown could not sensitise TRAIL and Smac59 resistant cell lines

In TRAIL resistant cell line HSC3M3, XIAP knockdown using siRNA did not sensitise the cells to TRAIL. Slight loss of cell viability was observed in the knockdown cells but this was the same with the control siRNA transfected cells (Figure 5.9) indicating that this was due to the transfection process itself. These results suggested that XIAP overexpression is not responsible for resistance to TRAIL-induced apoptosis.

Our data is different from some previous studies that showed that knockdown of XIAP and cIAP-1 increased sensitivity of prostate cancer cells to TRAIL (Dai et al., 2009b), and that XIAP knockdown using siRNA was able to sensitise chronic lymphocytic leukaemia resistant cells to TRAIL-mediated apoptosis (Frenzel et al., 2011). However, our results are in agreement with the finding that chemotherapeutics that induce cell death through the mitochondrial pathway required only inhibition of XIAP for sensitisation, whereas chemotherapeutics that induce cell death through multiple apoptotic pathways required inhibition of cIAP-1, cIAP-2 and XIAP (Greer et al., 2011). Furthermore, Smac mimetics enhanced TRAIL activity in breast, prostate and colon cancer cell lines by targeting XIAP and cIAP-1 (Lu et al., 2011). Combined knockdown of XIAP and cIAPs would be a logical next step to understand how IAPs are involved in TRAIL and Smac mimetics induced apoptosis in HNSCC cell lines.

In the HSC3M3 cell line, XIAP knockdown was able to increase sensitivity to Smac59 (Figure 5.9) suggesting that XIAP plays a role in Smac59 induced apoptosis. It is notable that we were not able to establish XIAP knockdown stable cells from this cell line which may indicate that XIAP expression in this cell line is essential for its survival.

In the Smac59 resistant UMSCC11B cell line, XIAP knockdown using siRNA or by establishing stable XIAP knockdown cells could not sensitise the cells to Smac59. Which member of the IAPs family is essential for Smac mimetic-induced apoptosis remains controversial. Some studies have shown that XIAP plays a critical role in inhibiting apoptosis induction by Smac mimetics and that removal of cIAP1/cIAP2 by Smac mimetics is not sufficient for TNF-α-dependent apoptosis induction (Lu et al., 2008). Other studies however showed that some cancer cells (NSCLC) mediate resistance to Smac mimetic by up-regulating cIAP2 (Petersen et al., 2010). We suggest that the mechanism of TRAIL and Smac mimetic induced apoptosis is cell type dependent and is affected by which apoptotic machinery is active in each cell. Understanding the biology and the genetic phenotype (using gene and miRNA expression profiling) of each cell could help to predict how each cell responds to a specific therapeutics.

In summary, this study showed that, TRAIL and Smac59 were effective in killing all HNSCC cell lines tested suggesting potential value in treatment of head and neck cancers. Caspase-8 expression seemed to be an important factor in predicting sensitivity of HNSCC cell lines to TRAIL. TNF-α expression was a predictor for Smac59 sensitivity in HNSCC cell lines and was able to sensitise the Smac59 resistant cells. Bcl-2 overexpression was able to protect the TRAIL sensitive cells against TRAIL induced apoptosis. Caspase-8, TNF-α, EFGR and Bcl-2 are potential biomarkers for TRAIL and Smac mimetic sensitivity in HNSCC cell lines. Further investigations including more HNSCC cell lines as well as animal studies are necessary to take these two agents towards clinical trials for treating HNSCC patients.

7.1.10 Biomarkers for response of HNSCC to treatment

Surgery alone or combined with radiotherapy and/or chemotherapy is the most common treatment modality for HNSCC patients. Many patients fail these treatments and develop recurrences or metastases as well as suffering the unpleasant side effects of chemo/radiotherapy. Identification of patients who are likely to

respond to such treatments before starting the treatment would spare unnecessary suffering and achieve more successful outcome. Radiotherapy and chemotherapy induce cell death by causing damage to DNA that cannot be repaired and this activates apoptotic pathways in cancer cells and results in their elimination. Some cancer cells escape this process either by escaping check point for DNA damage repair or by activating DNA damage repair machinery.

In this project the expression of a number of genes involved in DNA damage repair and tumour hypoxia was investigated in tissues from 150 HNSCC patients using immunohistochemical staining. Expression of these genes was tested for association with survival and treatment outcome. In this patient cohort, males were more frequently affected than females and age range was from 29 to 91 years with median of 60 years. This finding is in accordance with the previous reports about United Kingdom patients (Bonner et al., 2006).

Moderate tumour differentiation was predominant in carcinomas of all sites except tonsil and pharynx carcinomas in which poor differentiation was more common. TNM stage 4 was common in carcinomas of lateral tongue, retromolar region, alveolus, tonsils and pharynx. Stage 3 and stage 4 were equally prevalent in patients with floor of the mouth carcinomas. Stage II and stage IV were comparable in buccal mucosa cases. This lack of stage 3 may be because buccal carcinomas involve skin early and this makes them T4 at a small diameter. Stage 1 was common in patients with lip carcinomas.

7.1.11 TNM stage, lymph node metastases, site of tumour and treatment were associated with survival and patient's outcome.

In this study, cases with lymph node metastases at diagnosis and high TNM stage had poorer overall survival and disease free survival than cases without. These findings are in agreement with previous reports of head and neck carcinomas which showed that the most important prognostic factors are site and TNM stage and that patients with tumours that have spread to lymph nodes have poorer survival and that the extranodal spread reduced five year survival by more than half (Mehanna et al.,

2010). Furthermore, other studies showed that the number of positive lymph nodes and total number of excised lymph nodes was the only significant predictor of outcome in patient receiving adjuvant radiotherapy and was the only independent predictor of overall and disease free survival (Gil et al., 2009). All these findings demonstrate the importance of proper screening of lymph nodes involvement and importance of radical treatment decisions and confirm our series as representative.

Site of origin may also affect the survival of the patients due to proximity to vital organs or inaccessibility to radical treatment. In this study, overall survival was not affected by site of origin however; floor of mouth carcinomas had the least disease free survival. Carcinoma of base of the tongue is known to have low survival rates (Zhen et al., 2004), and lip carcinomas to have the best survival rates (Mehanna et al., 2010).

Patients treated with surgery followed by radiotherapy had shorter disease free survival than patients treated only with surgery while there was no difference in the overall survival of both groups. This is likely to be because adjuvant radiotherapy is usually used in more advanced tumours or in tumours with positive margins and such tumours will have more chances to develop recurrence and metastases.

7.1.12 ERCC1 expression was not associated with patients survival but was significantly associated with treatment outcome

ERCC1 expression (staining intensity or percentage of positive cells) had no significant association with overall survival or disease free survival in the study patients despite those carcinomas with low percentage of ERCC1 expressing cells achieving a better disease free survival than cases with high percentage of ERCC1 expressing cells. Our data was in accordance with previous reports which showed that ERCC1 expression was not correlated with survival in HNSCC (Lima et al., 2012) but differ with the finding that ERCC1 expression correlated with long survival in non small cell lung carcinoma patients who did not receive

chemotherapy (Lee et al., 2008). However, non small cell carcinoma is different than HNSCC in pathogenesis presentation and prognosis.

Our results showed ERCC1 expression (staining intensity) was significantly associated with patient's response to treatment as ERCC1 negative cases showed better outcome than ERCC1 positive cases. ERCC1 is involved in both recognition of the damage and excision of the damaged part of DNA (Hoeijmakers, 2001) and its expression has been correlated with resistance of HNSCC patients to cisplatin-based chemotherapy (Handra-Luca et al., 2007). Furthermore, our results showed that ERCC1 expressing cases recurred more frequently than ERCC1 negative cases suggesting that ERCC1 enhances the survival of cancer cells. These findings imply the role of ERCC1 in the repair of radiotherapy induced DNA damage and suggest that ERCC1 could be used as a prognostic marker for response to radiotherapy. However, our study did not have sufficient power to maintain ERCC1 as independent prognostic factor in multivariate analysis.

7.1.13 HIF-1α expression in HNSCC tissues was significantly associated with disease free survival and patients response to treatment

Under normoxic conditions HIF-1α degrades rapidly but under hypoxic conditions, HIF-1α accumulates in the nucleus, dimerizes with HIF-1β subunit and activates transcription of more than 100 target genes that are responsible for inducing cell proliferation, angiogenesis, local invasion and metastasis. Increased levels of HIF-1α in tumours has been associated with poor clinical outcome in different types of cancers including breast, cervical and endometrial cancers (Semenza, 2010).

In this study, HIF-1α was found to be expressed in HNSCC tissues and its expression (staining intensity or percentage of positive cells) was significantly associated with disease free survival as HIF-1α positive cases had poorer disease free survival suggesting that HIF-1α promotes the survival characteristics of cancer cells leading to tumour survival. Our data is in agreement with previous findings that low HIF-1α level was significantly related to disease free survival in T2-staged

oral tongue cancer (Roh et al., 2009) and that HIF-1α expression was a significant adverse prognostic factor, locoregional control and cancer specific survival of HNSCC treated with primary radiotherapy (Silva et al., 2007).

In our study, HIF-1α expression (staining intensity or percentage of positive cells) was significantly associated with poor outcome of the patients treated with surgery followed by radiotherapy in univariate analysis but it did not retain its significance in multivariate analysis, may be because our patient group is retrospective and heterogeneous for treatment. These findings suggested that HIF-1α could be a marker for survival and for outcome of patients to treatment. However, other studies found that basal HIF-α expression levels are not predictive of radiosensitivity of human lung cancer and HNSCC cell lines (Schilling et al., 2012).

7.1.14 PTEN expression in HNSCC tissues was significantly associated with overall survival and response of patients to treatment

In this study, PTEN expression was generally weak and was identified as both cytoplasmic and nuclear but only nuclear expression was scored. Cytoplasmic PTEN expression was previously correlated with histological grade and biological behaviour of oral squamous cell carcinoma; PTEN expression was stronger and more frequent in well differentiated carcinomas, reduced in less differentiated tumours and was completely absent at the invasive front (Squarize et al., 2002).

In our study PTEN expression (percentage of positive cells) cases was significantly associated with overall survival as cases with high percentage of PTEN expressing cells had poorer overall survival than cases with low percentage of PTEN expressing cells. It was also significantly associated with patient's outcome to treatment as 55% of low PTEN expressing carcinomas responded better to treatment and 71% of high PTEN expressing tumours had a poorer response to treatment. PTEN plays a critical role in the DNA damage repair and cells deficient in PTEN have defective double strand break repair (Ming and He, 2012). So

carcinoma cells with low PTEN will not be able to repair radiotherapy-induced DNA damage and will lead to cell death and tumour elimination.

The roles of PTEN expression in patient survival and response to treatment have been discussed in previous studies. Ming and He showed that PTEN expression played an important role in the response of cancers to radiotherapy as reduced levels of PTEN were associated with radioresistance and hence poor treatment outcome (Ming and He, 2012). Snietura and co-workers found that patients with high PTEN expression had a favourable outcome after accelerated postoperative radiotherapy, compared to patients with low PTEN expression (Snietura et al., 2012).

Our results seems to contradict these previous findings, however, all previous studies scored tested expression of PTEN as a simple positive or negative, which in our study was not significantly associated with treatment outcome. In our study we tested the percentage of positive cells within the PTEN expressing tumours which provide a more informative measure in PTEN expression analysis in relation to survival and response outcome. Testing these findings at a larger scale study in patients treated with radiotherapy alone is necessary to further determine the role of PTEN expression in HNSCC response to radiotherapy as the numbers in our study was relatively small and our patient group was heterogeneous to treatment.

7.1.15 Ku80, Rad51 and XRCC1 expression in HNSCC tissues were not significantly associated with survival or response of patients to treatment

Ku80, Rad51 and XRCC1 are important proteins involved in DNA damage repair and their expression has been extensively studied and they have been correlated with survival, locoregional control and response to different treatments in different tumour types.

Ku80 overexpression in HNSCC was highly predictive of loco-regional failure and death (Moeller et al., 2011) and in vitro studies have shown that Ku80

overexpression correlated with radiation resistance in HNSCC cell lines (Chang et al., 2006) and that silencing Ku80 using small interfering RNA enhanced radiation sensitivity in lung, pancreas, oesophageal, prostate and breast cancer cell lines (Nimura et al., 2007). In our study Ku80 was expressed in all HNSCC cases at different levels so associations between its expression and survival or patient's outcome could not be determined. However, carcinomas with high percentage of Ku80 expressing cells had poorer overall survival and disease free survival than carcinomas with low percentage of Ku80 expressing cells.

Rad51 overexpression has been reported to increase resistance of mammalian cells to ionizing radiation (Vispe et al., 1998). Antisense inhibition of mouse Rad51 enhanced radio-sensitivity of mouse embryos to ionizing radiation. (Bernstein et al., 2002). Head and neck cancer patients with high Rad51 protein level in their pre-treatment tumour biopsies demonstrated poor cancer-specific survival rates than patients with lower Rad51 levels (Connell et al., 2006). Similarly, elevated expression of Rad51 was associated with poor prognosis in esophageal squamous cell carcinoma (Li et al., 2011). In this study, overall survival and disease free survival in both Rad51 positive and negative carcinomas were not significantly different. However, carcinomas with high percentage of Rad51 expressing cells had poorer overall survival and disease free survival than carcinomas with low Rad51 expressing cells in consistent with previous studies. Rad51 expression (staining intensity or percentage) was not associated with patient's response to treatment.

High levels of XRCC1 mRNA has been detected in the blood of HNSCC patients (Schena et al., 2012). However, other studies showed that XRCC1 expression is lower in HNSCC patient in North India than healthy controls (Kumar et al., 2012). High XRCC1 protein expression in HNSCC is associated with poor survival, especially in patients receiving chemoradiation (Ang et al., 2011). In our study, XRCC1 was highly expressed in almost all HNSCC tissues and it was not possible to correlate XRCC1 expression with survival or patient response to treatment.

In summary, this study show that expression of DNA damage repair genes and tumour hypoxia affect patient survival and response to treatment in particular to radiotherapy. ERCC1, HIF-1α and PTEN are promising biomarkers for HNSCC survival and treatment outcome. In this study, Ku80, Rad51 and XRCC1 were not significantly associated with patient survival or response to treatment.

7.1.16 HPV status was associated with patient survival and response to treatment

In this study, eight out of 35 tonsil and pharyngeal carcinomas were p16 positive and six of these were HPV positive indicating that not all carcinomas arising in oropharyngeal areas are HPV related and that p16 is a good predictor of HPV positivity. P16 is commonly silenced in HPV negative carcinomas by multiple mechanisms, including mutation, deletion, and hypermethylation. In contrast, p16 is overexpressed in HPV positive oropharyngeal carcinomas because of E7-mediated pRb loss of feedback control leading to a paradoxical upregulation of p16 (Howard and Chung, 2012).

As expected, patients with HPV positive carcinomas had a better overall survival than patients with HPV negative carcinomas and HPV positivity was associated with better response to treatment. This finding is in consensus with the previous findings about oropharyngeal carcinomas. In 2010, Ang et al. in their retrospective study of patients treated with fractionation radiotherapy, found that of 63.8% of patients with oropharyngeal cancer had HPV-positive tumours; these patients had better 3 year rates of overall survival among patients with HPV negative tumours and that HPV status was a strong and independent prognostic factor for survival among patients with oropharyngeal cancer (Ang et al., 2010). In another prospective study on oropharyngeal carcinomas treated with chemotherapy, HPV was detected in tissues of 40% of patients with HNSCC of the oropharynx or larynx by in situ hybridization and PCR and that patients with HPV positive tumours had higher response rates after induction chemotherapy than HPV negative tumours (Fakhry et al., 2008).

There is a growing interest in understanding the underlying mechanisms that make HPV positive tumours more sensitive to treatment. Among these interests is the effect of HPV infection on the expression of DNA damage repair genes. In a previous study by Moeller et al., Ku80 expression was found to be a DNA repair-related biomarker of radiation treatment failure particularly in HPV negative HNSCC (Moeller et al., 2011). Another study by Mani et al. in 2011, showed that HIF-1α as a marker of hypoxia was less expressed in HPV positive tumours indicating that HPV positive tumours are less hypoxic and this could be a mechanism to explain their radiosensitivity (unpublished data). In our study, no association was detected between HPV status and expression of ERCC1, HIF-1α, Ku80, PTEN, Rad51 and XRCC1 proteins. Unfortunately the small number of HPV positive carcinomas included in the study series prevents definitive analysis and further work to explore potential associations will require many more cases specifically selected to test this hypothesis. One area of interest and related to this work is that HPV infection modulates apoptosis in HPV positive tumours (Lagunas-Martinez et al., 2010). How HPV would affect the expression of DNA damage repair genes which in turn will affect the apoptosis induction following radiotherapy treatment need to be investigated in a prospective study including large number of cases treated primarily with radiotherapy.

7.1.17 Conclusions

In conclusion, biomarkers predicting response of HNSCC to treatment are essential to improve curability. Biomarkers that could determine preoperative prognosis and treatment response through routine tumour biopsy is the ultimate achievement. In this study we have investigated the response of HNSCC cell lines to two novel therapeutic drugs (TRAIL and Smac59) and we investigated candidate biomarkers which could predict response of HNSCC cell lines to these novel targeted agents. Furthermore, we investigated the expression of genes involved in DNA damage repair and tumour response to hypoxia in tissues of HNSCC patients in a trial to find whether the expression profile of these genes could be correlated to the response of the patients to treatment.

The main findings of the apoptosis study:

- TRAIL and Smac59 was effective in killing HNSCC cell lines suggesting them as potential therapeutics for HNSCC treatment. Head and neck cancer cell lines can be grouped based on sensitivity/resistance to TRAIL and Smac59.
- Protein and mirRNA biomarkers could predict response of HNSCC cell lines to TRAIL and Smac59.
    - ♦ Importantly an association was found between TNF-α expression and sensitivity to Smac59, adding rhTNF-α to Smac59 sensitised Smac59 resistant cells suggesting TNF-α as a strong predictive marker for Smac59 sensitivity.
    - ♦ Caspase-8 overexpression was associated with sensitivity to TRAIL and could be a marker for TRAIL sensitivity in tumour cells that use the extrinsic pathway for apoptosis induction.
    - ♦ Several miRNAs were differentially expressed in TRAIL/Smac59 cells that could be important biomarkers for

TRAIL and Smac59 sensitivity, mir-9 seemed to be a promising predictor for Smac59 resistance.

- Bcl-2 overexpression protected HNSCC cell lines from TRAIL induced apoptosis but it could not protect them from Smac59 induced apoptosis.
- TRAIL and Smac59-mediated depletion of XIAP seems to be a consequence rather than the cause of caspase dependent induction of apoptosis.

The main findings of our clinical series:

- Lymph node metastasis was associated with poor overall survival and disease free survival as well as bad response to treatment in HNSCC patients.
- ERCC1 expression was significantly associated with treatment outcome in HNSCC patients suggesting that ERCC1 expression could be a biomarker for response of patients to treatment.
- Low HIF-1α expression was significantly associated with disease free survival and response of patients to combined surgery and radiotherapy treatment making HIF-1α a strong candidate as a prognostic biomarker.
- High PTEN expression indicated reduced overall survival and worse response of patients to treatment and this has potential as a prognostic biomarker in HNSCC.
- Patients with HPV positive carcinoma had a better overall survival and responded better to treatment.
- No association was detected between HPV positivity and expression of ERCC1, HIF-1α, Ku80, PTEN, Rad51 and XRCC1 proteins.

7.1.18 Future work

Future in *vitro* and in *vivo* studies will determine the potential of TRAIL and Smac mimetics and their modulators alone or in combination with radiotherapy and/or chemotherapy for personalised treatment of head and neck cancers.

- Investigate the role of miR9 in Smac59 sensitivity and the involvement on NF-κB in this process.
- Investigate the underlying mechanisms of TNF-α sensitisation of Smac59 resistant cells to Smac59.
- Investigate the effect of specific caspase inhibitors on IAPs expression following TRAIL and Smac59 treatment.
- Caspase-8, Smac/DIABLO, Mcl-1 and cFLIP have shown differential expression in TRAIL and Smac59 sensitive and resistant cell lines. Further validation of these agents and their potential use as biomarkers is needed.
- Investigate the effect of combined XIAP and cIAPs knockdown on TRAIL and Smac59 sensitivity.
- Animal studies to test the in vivo effect of TRAIL and Smac59 on viability of HNSCC xenograft.
- Validation of ERCC1, HIF-1α, Ku80, Rad51, PTEN and XRCC1 as biomarkers for radiosensitivity via a prospective study with patient treated with radiotherapy alone.
- Validation of these candidate biomarkers, including testing overexpression/knockdown effect on response to radiotherapy using cell lines and in vivo mouse models.

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